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FACTORS AFFECTING BASIDIOSPORE DEVELOPMENT OF PELLICULARIA FILAMENTOSA[1]

Asa C. Sims, Jr.

SUMMARY

Basidiospores occurred on hyphae arising from mycelial mats of *Pellicularia filamentosa* that had developed on potato broth amended with sucrose or maltose 10 days after transfer of those mats to alligator weed stems and roots. Of the sugar concentrations used, 5 g/l of sucrose or maltose was optimum for basidiospore development.

Basidiospores developed 30 days after mycelial fragments of isolates from basal semisynthetic agar were transferred to 2 per cent agar and maintained at 20°C. None developed at 15°, 25°, or 30°C.

Basidiospores developed on appressed hyphae of *P. filamentosa* on the inside of petri dish covers that were maintained over sterile distilled water for 10 days.

Basidiospores developed on the hyphae of mycelial mats of isolates that had been cultured previously on potato broth, amended with 5 g/l of sucrose, and maintained in growth tubes at a relative humidity of nearly 100 per cent.

Basidiospores developed on plants of cotton, alligator weed, wandering jew, potato, or sugar beet after the plants were inoculated with mycelial suspensions of certain isolates and maintained under bell jars at 20°C. Basidiospores were not observed when pieces of mycelial mats of the same isolates were inoculated on the same species of plants similarly maintained under bell jars at the same temperature.

Basidiospore development also occurred when 5 g/l or 20 g/l of sucrose were added to autoclaved plant juice extracts of potato and alligator weed.

Pellicularia filamentosa (Pat.) Rogers is an economically important plant pathogen with a wide suscept range. Some factors that affect basidiospore development were investigated so that single-basidiospore isolates could be obtained for later use in a study of the nature of parasitism.

Kotila[2] reported that basidiospores were found on the leaf blades and petioles of diseased sugar beet plants kept at approximately 100 per cent relative humidity and at 21°C. Hawn and Vanterpool[3] reported that basidiospores developed on isolates (from flax) placed on a rich nutritive substrate for 25–30 days and then transferred to 2 per cent agar. Exner[4] observed basidiospores when mycelial mats of the fungus were placed against rooted cuttings of alligator weed (*Alternanthera philoxerioides* (Mart.) Griech). These methods, plus modifications, were used in this study.

Two environmental regimes, A and B, were used. In regime A, mass isolates were cultured in different antecedent environments and then transferred to other environments. In regime B, mass isolates were transferred directly from stock cultures to modified environments.

[1] Accepted for publication March 13, 1956.

Paper No. 582, Department of Botany and Plant Pathology, Ohio State University. Part of a thesis presented to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the Master of Science Degree. The author is grateful to Dr. C. C. Allison and Dr. Patricia Allison for their helpful suggestions throughout the course of the investigation.

[2] Kotila, J. E. 1947. Rhizoctonia foliage blight of sugar beets. Jour. Agr. Res. 74: 289-314.

[3] Hawn, E. J., and T. C. Vanterpool. 1953. Preliminary studies on the sexual stage of Rhizoctonia solani Kühn. Canad. Jour. Bot. 31: 704-710.

[4] Exner, Bernice. 1953. Comparative studies of four Rhizoctonias occurring in Louisiana. Mycologia 45: 698-719.

METHODS AND EXPERIMENTAL RESULTS.—*Regime A.*—Isolates of *P. filamentosa* were placed on potato broth containing different concentrations of maltose or sucrose. Mycelial mats that developed were transferred to alligator weed stems and roots in the manner described by Exner.[4] Basidiospores formed, 10 days after transfer, on mycelial mats of most of the isolates that had been on potato broth amended with 5 g/l sucrose or 5 g/l maltose. Of the sugar concentrations used, 5 g/l was optimum for basidiospore development (Table 1).

Isolates were cultured for 30 days on basal semisynthetic agar and on malt agar, both maintained at 15°, 20°, 25°, and 30°C. Mycelial agar discs, 5 mm in diameter, were transferred to 2 per cent agar in petri

TABLE 1.—*Number of isolates of P. filamentosa on which basidiospores developed after their transfer to alligator weed stems and roots in flasks from potato-broth cultures containing different concentrations of sucrose or maltose*

Kind of sugar added	Number of isolates (of 28 tested) that sporulated following transfer from potato broth containing the indicated amounts of added sugar (g/l)				
	0	5	10	15	20
Sucrose	0	19	0	0	5[a]
Maltose	0	16[b]	0	2[c]	7[d]

[a] All also recorded under 5 g/l sucrose.

[b] Includes 1 that sporulated under no other conditions, 10 otherwise recorded only under 5 g/l of sucrose, and 1 under 5 g/l and 20 g/l sucrose.

[c] Basidiospores formed under no other conditions.

[d] Includes 3 in which basidiospores formed under no other conditions; 1 otherwise recorded only under 5 g/l sucrose; 2 otherwise recorded under 5 g/l of sucrose and 5 g/l of maltose; and 1 otherwise recorded under 5 g/l sucrose, 20 g/l sucrose, and 5 g/l maltose.

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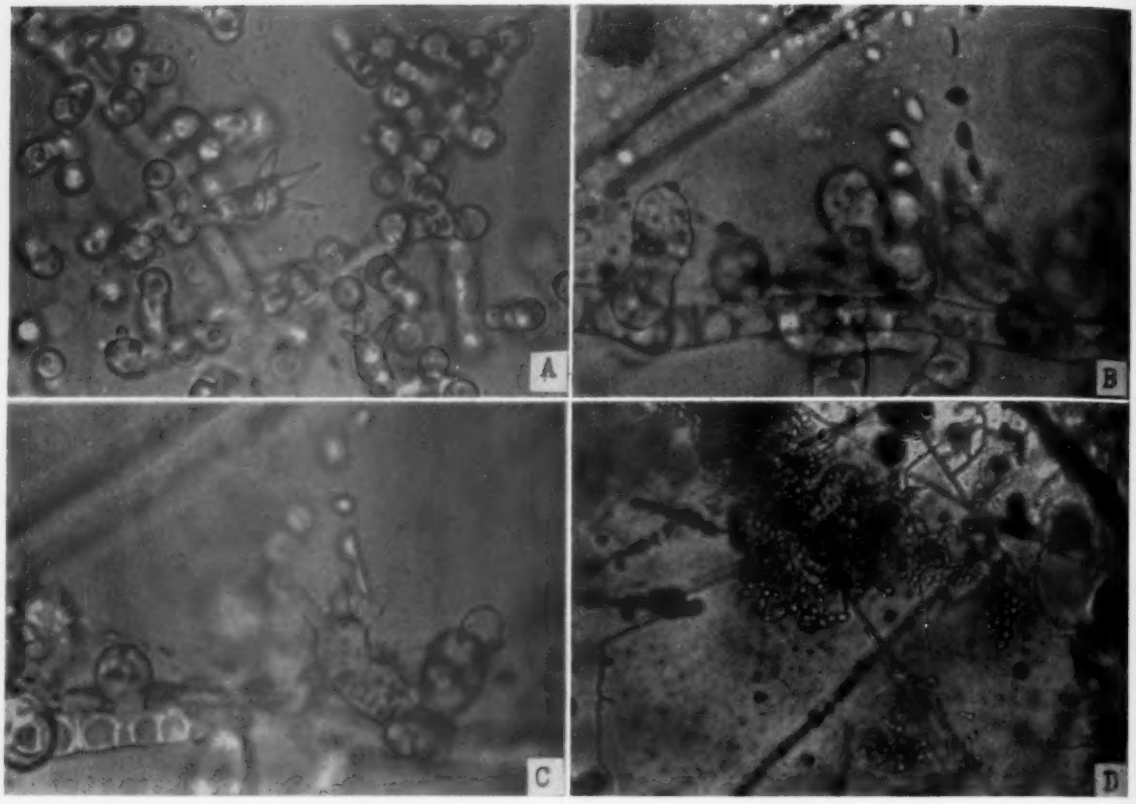

FIG. 1. A) Sterigmata and basidia from aerial hyphae of *Pellicularia filamentosa*. 220 ×. B) Discharged basidiospores from basidia of *P. filamentosa*. 500 ×. C) Basidium with fully expanded sterigmata. 500 ×. D) Barrel-shaped cells arising from subsurface mycelium of *P. filamentosa* growing in 2 per cent agar. 100 ×.

dishes. Basidiospores formed 30 days later only on those cultures previously maintained on basal semisynthetic agar at 20°. Basidiospores were not observed on isolates maintained on malt agar at any temperature.

Isolates were cultured on basal semisynthetic agar in petri dishes until hyphae were appressed on the inside of the petri dish cover. The covers, with the appressed hyphae, were placed over sterile distilled water maintained at 20°C. Basidiospores appeared after 10 days (Fig. 1, A, B, C).

Basidiospores formed when mycelial-mat pieces of isolates (originally from cotton) growing on potato broth amended with 5 g/l of sucrose were placed in growth tubes maintained at a relative humidity of about 100 per cent and at 20°C.

Regime B.—Basidiospores did not develop on cotton, tomato, alligator weed, wandering jew, potato, or sugar beet plants inoculated with stock mycelial-mat pieces of various isolates and maintained at 20°C.; nor were they observed when similarly treated plants were placed under bell jars. When plants were inoculated with mycelial suspensions of the same isolates, basidiospores formed on all plants, with the exception of tomato, covered with bell jars. No basidiospores occurred on uncovered plants although disease symptoms developed.

Basidiospores developed on isolates growing in autoclaved plant-juice extracts of potato and alligator weed. Basidiospores were formed also when a broth containing equal parts of potato and alligator weed extracts was used. These extracts were amended with different amounts of sucrose (0, 5, 10, 15, or 20 g/l) and kept at 20°C. Generally, basidiospores were found in all cultures growing in extracts with the exception of those containing 10 or 15 g/l or sucrose.

No basidiospores developed 60 days after isolates were transferred from stock potato-dextrose agar cultures to test tubes containing yeast-extract filter-paper agar, yeast-extract agar, or filter-paper agar made with purified agar. Colonies were small, and sclerotia and barrel-shaped cells were noted (Fig. 1, D).

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EFFECT OF SOIL TEMPERATURE AND GREEN-MANURE AMENDMENT OF UNSTERILIZED SOIL ON PARASITISM OF RHIZOCTONIA SOLANI BY PENICILLIUM VERMICULATUM AND TRICHODERMA SP.[1]

Michael G. Boosalis

SUMMARY

Rhizoctonia solani Kühn added to unsterilized field soil was parasitized by *Trichoderma* sp. and by *Penicillium vermiculatum* Dang. *Trichoderma* sp. was isolated from about 60 per cent of the parasitized Rhizoctonia hyphae, and *P. vermiculatum* was obtained from about 20 per cent of the invaded host hyphae. At a soil temperature of 28°C, about 18 and 8 per cent of the hyphae of *R. solani* in green-manure amended soils and in unamended soils, respectively, were parasitized by the above listed fungi. Not over 40 per cent of the unparasitized Rhizoctonia hyphae in both soil treatments remained viable after an 8-week sojourn in soil. In a parallel experiment, in which the soil temperature was kept at 18°C, *R. solani* was not appreciably parasitized by soil-inhabiting fungi. In this case, about 60 per cent of the unparasitized host hyphae from the unamended and green-manure-amended soils retained their viability.

Rhizoctonia solani was considerably more susceptible to *P. vermiculatum* when cultured on media having 20 g dextrose per liter than when grown on media containing only 10 g dextrose per liter.

Although some isolates of *R. solani* were more resistant to *P. vermiculatum* than were others, none of the 30 cultures of *R. solani* tested were highly resistant. *P. vermiculatum* vigorously parasitized an isolate of *R. solani* that in turn is parasitic on several other fungi.

Hyphae of *R. solani* are parasitized by *P. vermiculatum* by means of penetration pegs developing from mycelium in contact with the host hyphae.

Infestation of unsterilized soil with *P. vermiculatum* did not provide biological control of Rhizoctonia-incited damping-off and seedling root rot of peas.

A "hot-water method," involving submersion of infested material for 1.5 minutes in water at 75°C, was found to be an effective method for the isolation of *Streptomyces* spp., *P. vermiculatum*, and other species of *Penicillium*.

Circumstantial evidence has been accumulated to support the belief that soil-inhabiting fungi parasitic on other fungi play a part in the establishment and in the persistence of *Rhizoctonia solani* Kühn in the soil. Although Weindling (5) and Warren (4) definitely adduced that *Trichoderma lignorum* (Tode) Harz and *Papulospora* sp. parasitize *R. solani* growing in various synthetic media and in sterilized soil, there is a dearth of evidence to indicate that this fungus is parasitized in unsterilized soil. The purpose of this study was to ascertain whether *R. solani* added to unsterilized soil is parasitized by soil fungi and to make concurrent preliminary investigations on the effect of soil temperature and of green-manure soil amendment on the incidence of parasitism.

MATERIALS AND METHODS.—A culture of *R. solani* isolated from soybean (*Glycine max* (L.) Merrill) roots and shown to incite damping-off and root rot of soybeans and peas (*Pisum sativum* L.) was used throughout the study. A composite mixture of soil, Sharpsburg type pH 6.6, collected from several commercial soybean fields in the vicinity of Lincoln, Nebraska, was dispensed into galvanized flats, $2 \times 1.5 \times 0.5$ ft., each of which held 50 lb. Inoculum was increased on medium containing 100 parts sand, 1 part ground maize meal, and 13 parts water by weight. The unsterilized soil was infested with *R. solani* by adding and mixing thoroughly 15-day-old inoculum at the rate of 800 g/50 lb. of soil. The soil temperature for one set of flats was maintained at 18°C ± 1° and for the second series of flats at 28°C ± 2°. Soil moisture for both temperature treatments was regulated to about 35 per cent of its moisture-holding capacity. Green manure, consisting of soybean stems and leaves, was dried, ground, and mixed at the rate of 2 per cent on a dry-weight basis with soil contained in half of the flats. This treatment is referred to as the green-manure amendment. Concurrently the soil was infested with the prescribed inoculum. The 2 treatments for each of the 2 soil temperatures of 18° and 28°C included 1) 4 flats of soil artificially infested with *R. solani* and 2) 4 flats same as 1 plus green manure. Eight weeks after the soil was infested, 50 0.5-g random samples of the original Rhizoctonia inoculum, which was easily detected in the soil by the presence of sand, were removed from each replicate of the amended and unamended soils kept at 18° and 28°C. The inoculum from each treatment was rinsed separately in tap water until the mycelium was relatively free of soil and debris. The washed inocula from all replicates of a given treatment were then mixed. Several random samples were taken from the 4 mixtures and examined microscopically. Over 400 Rhizoctonia hyphae from each soil treatment maintained at 18° and at 28°C were examined for fungus parasitism.

Three methods of isolation were used for obtain-

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The author is indebted to Dr. K. B. Raper and Mr. M. L. Fuller for their valuable help in identifying *Penicillium vermiculatum*.

ing pure cultures of fungi associated with *R. solani*. The "trap-fungus culture method" consisted of transferring washed fungus hyphae (from each soil treatment) to 6-day-old Petri dish cultures of *R. solani* grown on a mixture of maize meal and sand. The resulting fungi growing on the Rhizoctonia culture were isolated and tested for pathogenicity to the host fungus. For the "hot-water method" (1) of isolation, which is essentially a modification of Warcup's (3) partial-sterilization-by-steam technique, the washed Rhizoctonia inoculum was submerged for 1.5 minutes in water heated to 75°C and then plated out on 1.8 per cent water agar, pH 4.8. The heat treatment apparently destroys those soil-inhabiting fungi whose thermal death point is below 57°–55°C. The above 2 methods of isolating fungi were utilized primarily to determine whether soil particles and debris closely associated with unparasitized Rhizoctonia inoculum, "fungus sphere," contained fungi deleterious to *R. solani*. Fungi microscopically observed within hyphae of *R. solani* were isolated by means of a dilution technique. In this case, single parasitized Rhizoctonia hyphae were isolated from comminuted dilution suspension of the washed inoculum, transferred to 1.5 per cent water agar (pH 4.8), and incubated for 3 weeks at 27°C. Isolates obtained by this method were in turn tested for pathogenicity to *R. solani* grown on synthetic medium.

Unless stated otherwise, parasitic and antibiotic tests were made on potato-dextrose agar (PDA) containing infusion from 350 g potato, 20 g dextrose, 18 g agar, and distilled water to make a total of 1 liter. Small portions of the host and test fungus were simultaneously placed on opposite sides of Petri dishes containing 20 ml of PDA and incubated for 3 weeks at 27°C. Each test was replicated 5 times. All experiments reported herein were run twice.

EXPERIMENTAL RESULTS.—*Effect of 18°C soil temperature and of green-manure amendment on fungus parasitism of R. solani.*—The relatively low soil temperature of 18°C was not conducive to fungus parasitism of *R. solani*. Microscopic observations of Rhizoctonia mycelium from unamended and from green-manure-amended soil did not reveal an appreciable amount of parasitism. Furthermore, when the Rhizoctonia inoculum was comminuted in a Waring Blendor and dilution plates made, it was evident that about 55 per cent of the hyphae from both soil treatments were viable (Table 1).

The trap-fungus method of isolating fungi from the fungus sphere of Rhizoctonia yielded predominately isolates of *Trichoderma* sp. and several species of *Penicillium*. Parasitic tests indicated that about 60 per cent of the 100 isolates of *Trichoderma* sp. parasitized in some degree the hyphae of *R. solani*. Parasitic or antibiotic activity was not evident with any of the isolates of *Penicillium* spp. No attempt was made to ascertain the relative number of fungi associated with the Rhizoctonia inoculum from the unamended and green-manure-amended soils.

Isolates of *Streptomyces* spp. and *Penicillium* spp. were obtained by the hot water method of isolation from Rhizoctonia inoculum in both soil treatments. *Trichoderma* spp. were not isolated by this technique. Approximately 80 and 60 per cent of the 100 samples of Rhizoctonia inoculum from each of the green-manure-amended and unamended soils, respectively, yielded *Streptomyces* spp. Of these cultures of *Streptomyces* spp. isolated from both soil treatments, about 2 per cent were strongly antibiotic to *R. solani*. Parasitism of the host fungus was not detected, however, with any of the antibiotic isolates of *Streptomyces* spp. or with any of the other 140 isolates of *Streptomyces* spp.

None of the 20 isolates of 3 species of *Penicillium* obtained from the Rhizoctonia inoculum in either of the 2 soil treatments parasitized the host hyphae.

Apparently under the conditions of the experiment, green-manure-amended and unamended unsterilized soils having 35 per cent moisture and maintained at 18°C are not favorable for fungus parasitism of *R. solani*. The fact that over 50 per cent of the host

TABLE 1.—*Percentage of hyphae of Rhizoctonia solani parasitized by fungi when grown in unsterilized soil, with and without added green manure, maintained at 35 per cent of its water-holding capacity and incubated at 2 different temperatures*[a]

Treatment	Soil fungi[b]	Percentage of hyphae of *R. solani* parasitized by indicated fungi: *Trichoderma* sp.[c]	*Penicillium vermiculatum*[c]	Percentage of hyphae remaining viable[d]
Soil temperature: 18°C				
Unamended soil	0.0	0	0	60
Green-manure-amended soil	0.2	0	0	55
Soil temperature: 28°C				
Unamended soil	8	65	28	40
Green-manure-amended soil	18	60	30	20

[a] Quantitative and qualitative determination were made 8 weeks after the soil was artificially infested with *R. solani*.
[b] Percentage based on the average of 4 replicates with each replicate containing 100 hyphae.
[c] Percentage based on 400 parasitized hyphae of *R. solani* yielding *Trichoderma* sp. or *P. vermiculatum*.
[d] Percentage based on 400 unparasitized hyphae of *R. solani* that produced colonies when transferred to potato-dextrose agar.

hyphae were viable 8 weeks after the soil was infested indicated that the activities of soil organisms were not sufficiently harmful to kill the majority of host hyphae.

Effect of 28°C soil temperature and of green-manure amendment on fungus parasitism of R. solani.—Parasitism of *R. solani* was more prevalent in 28° soil than it was in soil kept at 18°C (Table 1). The highest incidence of parasitism occurred in green-manure-amended soil where 18 per cent of the host hyphae were invaded by fungi. In contrast, only about 8 per cent of the host hyphae in the unamended soil were parasitized by fungi. *Trichoderma* sp. was the predominant parasite isolated by the dilution method from 400 parasitized Rhizoctonia hyphae obtained from each soil treatment. Nearly 60 per cent of the parasitized hyphae contained *Trichoderma* sp. *Penicillium vermiculatum* Dang. was isolated from about 20 per cent of the parasitized Rhizoctonia mycelia recovered from the unamended and green-manure-amended soils. Fungi were not isolated from about 20 per cent of the hyphae of *R. solani* microscopically discerned to have been parasitized. In no case were *Trichoderma* sp. and *P. vermiculatum* obtained from the same parasitized hypha of *R. solani*. The ratio of *Trichoderma* sp. to *P. vermiculatum* isolated from the parasitized host hyphae in the unamended soil was about the same as for the green-manure-amended soil. Of the 40 cultures of *Trichoderma* sp. and of the 20 of *P. vermiculatum* isolated from host fungus in both soil treatments, all parasitized *R. solani* when tested *in vitro*.

Nearly 80 and 60 per cent of the host hyphae from the green-manure-amended and unamended soils, respectively, were not parasitized by fungi. Other factors, in addition to parasitism, are undoubtedly operating in the soil to incite injury to the host hyphae. This was evident by the fact that only 20 and 40 per cent of the unparasitized Rhizoctonia hyphae from the amended and unamended soil, respectively, had retained their viability.

Again, *Trichoderma* sp. was obtained by the fungus-trap culture method from nearly all the 100 samples of Rhizoctonia inoculum from the amended and unamended soils. Parasitic activity was detected for 30 and 20 per cent of the *Trichoderma* sp. isolated from the green-manure-amended and unamended soils, respectively. None of the *Pencillium* species isolated from the Rhizoctonia inoculum in either soil treatment was parasitic on Rhizoctonia.

The principal fungi isolated by the hot-water method from hyphae of *R. solani* in the 2 soil treatments were several species of *Penicillium*, of which the most frequently isolated was *P. vermiculatum*. This species of *Penicillium* was isolated in over 60 per cent of the 100 samples of Rhizoctonia inoculum from each of the 2 soil treatments. The vigorous hot-water method of isolation again eliminated most of the fungi, including *Trichoderma* spp. It was interesting to note that the thermal death point of an 18-day-old culture of *P. vermiculatum* that developed mature cleistothecia on PDA was 65°C.

Certain species of *Streptomyces* also withstood the heat treatment, consequently they frequently were isolated from the Rhizoctonia inoculum from the amended and unamended soils. Of the 200 cultures of *Streptomyces* spp. obtained from both soil treatments, only about 2 per cent were strongly antibiotic to *R. solani*. None of the *Streptomyces* spp., however, parasitized the host fungus *in vitro*.

The foregoing results indicate that a high soil temperature of 28°C stimulates parasitism of *R. solani* by *Trichoderma* sp. and by *P. vermiculatum* in both the green-manure-amended and unamended soils. Furthermore, with a soil temperature of 28°C, the incidence of parasitism of *R. solani* in the green-manure-amended soil was more than 50 per cent higher than in the unamended soil. Notwithstanding the stimulatory effects of high soil temperature and green-manure amendment, over 80 per cent of the host hyphae from any of the treatments were not parasitized by soil fungi. Under the conditions of the experiments, parasitism of *R. solani* does not appear to be a major factor affecting the survival of the pathogen in the soil. Survival of only 20 per cent of the host hyphae in the 28°C green-manure-amended soil indicates that 1 or more factors other than fungus parasitism impair the viability of the Rhizoctonia mycelium.

Effect of dextrose on parasitism of R. solani by Penicillium vermiculatum in vitro.—Exploratory studies disclosed that the amount of dextrose contained in the PDA medium has a profound effect on the incidence of parasitism of *R. solani* by *Penicillium vermiculatum*. The host fungus cultured on PDA having 20 g dextrose per liter (high-level-dextrose PDA) was severely parasitized by *P. vermiculatum*. The parasitic fungus covered the surface of a 9-mm Petri dish culture of *R. solani* in 16 days. An abundance of yellow cleistothecia was produced over the surface of the Rhizoctonia culture by the parasite. Nearly 60 per cent of the Rhizoctonia hyphae was invaded by hyphae of the parasitic fungus. In none of the 5 replicated cultures were all examined host hyphae parasitized by *P. vermiculatum*. This was true even with cultures that were kept for 40 days. *P. vermiculatum* grown alone covered the surface of the Petri dish substrate in less than 9 days.

The parasitic activity of *P. vermiculatum* was substantially reduced when the host fungus was grown on PDA having 15 g dextrose per liter (medium-level-dextrose PDA). The fungus parasite required about 18 days to grow over the surface of a Petri dish culture of Rhizoctonia. About 35 per cent of the hyphae of *R. solani* grown on this medium were parasitized by *P. vermiculatum*.

The incidence of parasitism was negligible on *R. solani* grown in PDA with only 10 g dextrose per liter (low-level-dextrose PDA). The hyphae of *P. vermiculatum* did not extend more than 2.5 cm over the Rhizoctonia culture in 40 days. Less than 3 per

cent of the host hyphae overgrown by the parasite were parasitized. In the control treatment, *P. vermiculatum* extended over the surface of the medium in about 13 days. Contrary to the abundant production of cleistothecia produced on high- and medium-level-dextrose PDA, the parasitic fungus developed relatively few sexual fruiting bodies on the low-level-dextrose PDA seeded with *R. solani*.

The parasitic activity of *P. vermiculatum* on *R. solani* cultured on Difco malt agar was comparable to that observed on PDA.

The effect of dextrose on the incidence of parasitism was studied also in Petri dishes divided into quadrants. Two opposite quadrants with low-level-dextrose PDA were seeded with *P. vermiculatum* and the other 2 opposite quadrants containing a high level of dextrose were seeded with *R. solani*. After incubation for 20 days at 27°C, *P. vermiculatum* had grown profusely over the low-level-dextrose PDA quadrants and had extended over the entire surface of the high-level-dextrose quadrants that had been seeded with *R. solani*. Over 40 per cent of the host hyphae were parasitized.

Results similar to the above were obtained when medium-level-dextrose PDA was substituted for the high-level-dextrose medium. In this case, however, the incidence of parasitism of *R. solani* grown on the medium-level-dextrose PDA was only 25 per cent of the hyphae.

When *P. vermiculatum* and *R. solani* were grown on high- and low-level-dextrose PDA quadrants, respectively, the parasitic fungus grew sparsely over the quadrants seeded with *R. solani*. Less than 5 per cent of host hyphae cultured on the low-level-dextrose quadrants were invaded by *P. vermiculatum*.

The high incidence of parasitism of *R. solani* grown on high-level-dextrose PDA cannot be ascribed entirely to the high osmotic pressure of the substrate. In this connection, *R. solani* was vigorously parasitized by *P. vermiculatum* on sand medium having only 0.3 per cent maize meal by weight. It appears that the kind and/or the amount of carbohydrate and perhaps the carbohydrate:nitrogen ratio of the medium are important factors affecting the incidence of parasitism.

Fungus host range of P. vermiculatum.—Of the 28 species of fungi belonging to 16 genera, only *R. solani* was parasitized by *P. vermiculatum* when tested *in vitro*. Fungi not parasitized by *P. vermiculatum* included *Alternaria* sp., *A. solani* (Ell. & G. Martin) L. R. Jones & Grout, *Aspergillus fumigatus* Fres., *A. niger* v. Tiegh., *Cephalosporium* sp., *C. gregatum* Allington & Chamberlain, *Chaetomium* sp., *C. globosum* Kuntze, *Fusarium oxysporum* Schlecht., *F. oxysporum* f. *lini* (Bolley) Snyd. & Hans., *Gibberella zeae* (Schw.) Petch, *Helminthosporium* sp., *H. avenae* Eidam, *H. sativum* Pam. King & Bakke, *Mucor* sp., 4 unidentified species of *Penicillium*, *Pythium* sp., *P. debaryanum* Hesse, *Phytophthora infestans* (Mont.) d B., *Rhizopus* sp., *Sclerotinia sclerotiorum* (Lib.) d B., *Sclerotium rolfsii* Sacc., *Trichoderma* sp., and *T. lignorum* (Tode) Harz.

None of the 30 cultures of *R. solani* isolated from 25 different host plants were highly resistant to *P. vermiculatum*. The incidence of parasitism, however, was considerably higher with some isolates of *R. solani* than with others. It was also interesting to note that *P. vermiculatum* was strongly parasitic to an isolate of *R. solani* that Butler (2) reported to be parasitic on several fungi.

Mode of parasitism of P. vermiculatum on R. solani.—*Penicillium vermiculatum* established internal parasitic mycelium in hyphae of *R. solani* by means of penetration pegs developing either from hyphae in direct contact with the host mycelium or from mycelium coiling around the host hypha. The number of parasitic hyphae observed within the host hyphae varied from 1 to 4, although the majority were invaded by a single hypha (Fig. 1). The extent of hyphal development within the mycelium of *R. solani* varied greatly. In some instances the entire hyphal thallus was gutted by the parasite, whereas in other instances the parasitic hyphae were confined within small portions of the host hyphae. The diameter of the parasitic hyphae was relatively small, usually less than 1/5 the diameter of the host hyphae (Fig. 1, D). Hyphal branching of the parasite occurred frequently and was observed regularly at the junction of hyphal branching of the host fungus (Fig. 1, A). Hyphae of *P. vermiculatum* that had grown out of the open ends of fragmented host hyphae were seen often. No case was seen where the parasitic fungus had penetrated through the hyphal wall of *R. solani* from the inside.

The parasitic activity of *P. vermiculatum* caused deterioration of the host protoplasm. Apparently, injury to *R. solani* is not elicited by toxic materials emanating from the hyphae of *P. vermiculatum*. Filtered culture extracts of the parasite cultured on any of several kinds of liquid and solid media incorporated in PDA caused little injury to *R. solani*. Similarly aqueous extracts of the hyphae of *P. vemiculatum* grown on PDA had no effect on the host fungus.

Biological control of Rhizoctonia damping-off and seedling blight of peas.—Studies were initiated to determine whether addition of *P. vermiculatum* to soil affords protection to peas against damping-off and seedling blight incited by *R. solani*. In this study, the peas were planted 10 days after the soil was infested with the prescribed inoculum. For all treatments, soil temperature was maintained at 28°C and soil moisture at 35 per cent of the water-holding capacity of the soil.

Nearly complete control against Rhizoctonia-incited damping-off and seedling blight of peas resulted when sterilized soil from commercial soybean fields was infested simultaneously with pure cultures of *P. vermiculatum* and of *R. solani*. About 60 per cent of the peas planted in sterilized soil infested with *R. solani* damped-off. Moreover, necrotic lesions were evident on 70 per cent of the surviving plants. Damping-off and seedling blight were negligible in soil

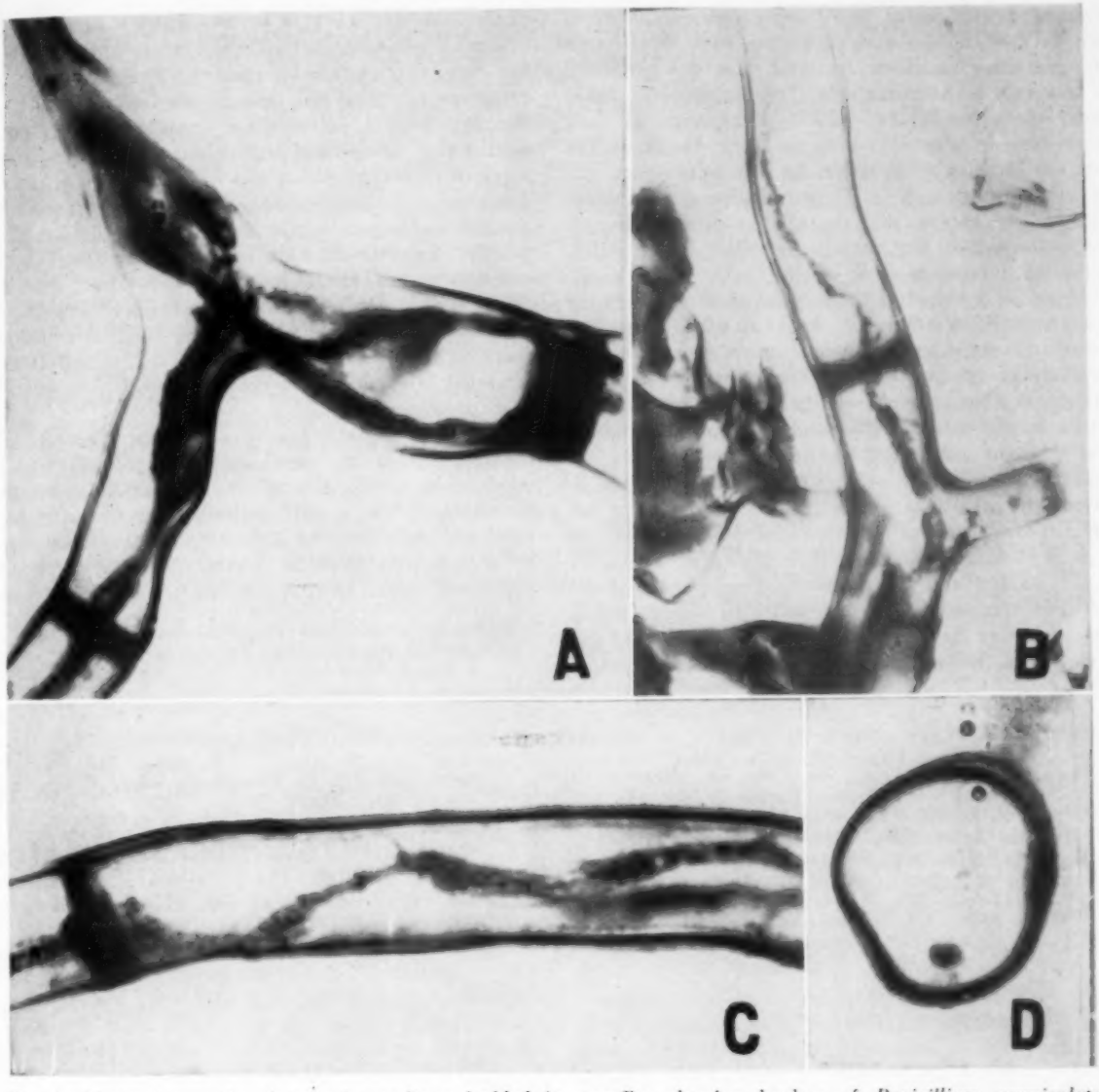

FIG. 1. Photomicrographs of stained mycelia embedded in paraffin, showing hyphae of *Penicillium vermiculatum* within the hyphae of *Rhizoctonia solani*. A) Branching hypha of *R. solani* invaded by *P. vermiculatum*. 1250×. B) Parasitized hypha of *R. solani* showing penetration through the septum. 1250×. C) Parasitized hyphal cell of *R. solani*. 1416×. D) Cross section of *R. solani* containing 2 hyphae of the parasite. 2500×.

infested only with *P. vermiculatum* and in the control treatment.

Biological control of *R. solani* in unsterilized soil infested with *P. vermiculatum* was not nearly as effective as in the sterilized soil. In this case, about 60 per cent of the peas damped-off in soil infested simultaneously with *R. solani* and *P. vermiculatum*. Of the surviving plants, nearly 50 per cent had seedling blight symptoms. The incidence of damping-off and seedling blight was also high in soil infested with *R. solani*. Peas planted in soil treated only with *P. vermiculatum* and in the control soil did not damp-off appreciably and they were relatively free of seedling blight.

DISCUSSION.—The factors contributing to the relatively high incidence of parasitism of *R. solani* are complex. Studies *in vitro* indicated that parasitism of *R. solani* by *Penicillium vermiculatum* was severest in potato-dextrose agar containing a high level of dextrose. It appeared that the high level of dextrose increased the incidence of parasitism by increasing the susceptibility of the host to the parasite. This leads to the assumption that the carbohydrate contained in the green-manure cuttings of peas, coupled with relatively high soil temperature, increases the susceptibility of *R. solani* to *P. vermiculatum*. Recently, Wright (6) reported an increased production of gliotoxin by *Trichoderma viride* in unsterilized soil sup-

plemented with wheat meal or with dried clover. It may be speculated that a substance, such as gliotoxin or some other antibiotic material, also was produced in relatively high amounts by these antagonistic fungi under these conditions. The high amounts of such antibiotics in the soil may in turn facilitate the internal invasion of *R. solani* by *Trichoderma* sp. and by other fungi such as *P. vermiculatum*. It is not assumed, of course, that the above-mentioned factors are the only ones that may be operating in the soil to stimulate parasitism of *R. solani*.

About 82 per cent of the hyphae of *R. solani* in the green-manure-amended soil kept at 28°C were not parasitized by fungi. It was shown, however, that only about 20 per cent of the unparasitized host hyphae retained their viability after a period of 8 weeks in the soil. These results lead to the theory that perhaps other factors are more important in reducing the viability of *R. solani* than is the injury incited by parasitism *per se*. Investigations were not pursued to determine what caused the unparasitized hyphae of *R. solani* to lose their viability. The work of Wright (6) and others, however, suggests that the increased production of antibiotics in supplemented soils may play some part in causing injury to *R. solani*.

Only the hot-water treatment method (1) especially designed for this study yielded isolates of *P. vermiculatum* from the soil. The efficacy of this method for obtaining certain species of *Penicillium* and of *Streptomyces* from the soil is ascribed to the high thermal death point of these organisms. It is noteworthy that the thermal death point of the ascongenous stage of *P. vermiculatum* was 65°C. Failure to isolate consistently *P. vermiculatum* from the soil by the soil-dilution method cannot be explained completely. It is possible, however, that the faster growing fungi of the soil inhibit the establishment of the slower growing fungi, such as *P. vermiculatum*, in culture. The urgent need for better methods of isolating fungi in investigations on antagonistic relationships in the soil is emphasized in these studies concerned with the isolation of *P. vermiculatum*.

Although biological control of damping-off and seedling blight of peas incited by *R. solani* was attained in sterilized soil infested with *R. solani* and *P. vermiculatum*, a comparable treatment in unsterilized soil gave little or no control. These results are in accord with those of others who have attempted biological control of root diseases in a similar fashion.

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AN INVITATION FROM THE 50TH ANNIVERSARY PROGRAM COMMITTEE

The Program Committee for the meeting to be held in celebration of the 50th Anniversary of the American Phytopathological Society extends to every person an invitation to submit suggestions for the program of that meeting. The Committee members will welcome suggestions of any nature; they particularly solicit ideas on symposia themes, symposia topics, and speakers.

Tentative plans call for 2 or 3 days of very significant symposia, with speakers drawn from the international scene. Your suggestions may be sent to any member of the Program Committee, which is composed of the following individuals:

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STEM INOCULATION OF PINTO BEAN WITH SOUTHERN BEAN MOSAIC VIRUS, A PROMISING METHOD FOR USE IN SCREENING CHEMICALS FOR ANTIVIRAL ACTIVITY [1]

J. W. Mitchell, W. H. Preston, Jr., and J. M. Beal [2]

SUMMARY

In attempts to devise a method of screening compounds for antiviral activity, a technique of producing localized infections with southern bean mosaic virus on hypocotyls of young Pinto bean plants was used. In tests with this method, at least 95 per cent of the inoculated areas consistently produced dark brown lesions that were readily detectable within 4–5 days.

Information on the possible use of this technique to detect the inhibiting effect of chemicals on virus multiplication was obtained from detailed studies of the site of multiplication and path of movement of the virus. Movement of the virus both upward and downward from the lesions was indicated by the formation of internal, but externally visible, longitudinal brown streaks. Detection of tissues in which the virus most readily multiplied and moved was achieved by use of 3 techniques: histological sectioning, a color test, and inoculation of indicator plants.

A tissue-dissection technique involving inoculation to indicator plants was used to detect the virus in the following composite samples: 1) the inoculated area and surrounding epidermis and cortex, 2) the remaining epidermis and cortical tissue, 3) the discolored portion of the pericycle and phloem (brown streak), 4) the remaining pericycle and phloem, and 5) the stele. The virus apparently entered the injured epidermal cells or outermost cortical cells, then moved slowly inward during the next few days. When the virus reached the phloem-pericycle tissues, movement was comparatively rapid upward and downward.

This information was useful in evaluating screening results, since chemicals in a suitable carrier (lanolin) were placed at the site of inoculation, near it, or at some distance from it. The absorption, translocation, and antiviral activity of these substances could then be evaluated.

Methods of screening chemicals for antiviral activity should be rapid and easily performed and should at least reveal compounds that reduce or prevent localized infections. Such methods would be even more useful if they also indicated whether the compounds are translocated in plants and if the compounds prevent systemic infections. Infection of localized areas on plant stems with a virus, as used in the present study, made possible such a method since the chemicals could be placed at the site of inoculation, near it, or at some distance from it; later their translocatability and their localized and systemic antiviral activity could be evaluated. The location of the virus in the plant tissues as indicated by histological and biological studies is important since, if the location is known, an attempt can then be made to apply the compounds in such a way that the infected tissue will absorb sufficient amounts to suppress or prevent further virus multiplication.

This paper describes 1) a method of producing stem infections in Pinto bean plants (*Phaseolus vulgaris* L.) with southern bean mosaic virus (SBMV) (*Marmor laesiofaciens* Zaum. & Harter); 2) the use of histological techniques to indicate tissues in which the virus multiplied most readily; 3) the use of triphenyltetrazolium chloride (2) and of inoculations to Pinto beans for determining the presence of virus in such tissues.

EXPERIMENTAL METHODS AND RESULTS.—*Stem inoculation.*—A scarification method was used for inoculating localized areas on stems of Pinto bean plants with SBMV. Inoculum was prepared by expressing juice from Black Valentine bean plants systemically infected with the virus. A large quantity of the inoculum was stored at 0°F in small vials each containing 0.2 ml of juice. Before inoculation, the juice was diluted with tap water (generally 1 part of juice to 2000 parts of water) so that it produced 90–100 per cent infection when used as inoculum.

Pinto bean plants used for these tests were grown in a greenhouse and selected for uniformity. At the time of inoculation, they possessed fully expanded primary leaves, and the first trifoliate leaf was beginning to unfold. Three drops (approximately 0.05 ml each) of inoculum were placed one above the other and approximately 5 mm apart on 1 side of the hypocotyl and directly beneath 1 cotyledon (Fig. 1), and then similar drops were placed in comparable positions under the opposite cotyledon. Approximately 1 mg of Carborundum was added to each drop. The tissue covered by each drop was then scarified by means of the tapered end of a glass rod (ground flat and 2 mm in diameter at the tip). The rod was moved with slight pressure in a circular motion 5–7 times over the area. The residual inoculum and Carborundum were washed off with tap water immediately after scarification of all 6 areas.

Within 4–5 days after inoculation, dark-brown sunken areas developed where the inoculations were made. Within 4–6 days after inoculation, internal but externally visible brown streaks developed; these extended downward from the inoculated areas sometimes

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to the ground line and upward to the second node (Fig. 2). Although test plants used in these experiments were grown under a wide variety of greenhouse conditions, consistently high percentages of infections (95 per cent or more of inoculated areas) have been obtained. Only during prolonged periods of cloudy weather did low percentages of infections occur.

It should be mentioned that Pinto bean plants inoculated on the leaves with SBMV usually develop local lesions. In preliminary studies it was noted that the virus did not spread readily in leaf tissue except when a lesion developed on a relatively large vein. In such instances it sometimes extended in both directions for about 0.5–1 cm from the point of inoculation. The extension of development of lesions on stems, however, was much more pronounced than that of lesions on leaves. Zaumeyer (6) has reported somewhat similar symptoms of SBMV in bean plants of the Blue Lake variety.

Chemical application. — After freshly inoculated areas on Pinto hypocotyls had dried, chemicals to be tested for antiviral activity were applied. Lanolin was melted and thoroughly mixed with a sufficient amount of the chemical to be tested to make a 1 per cent mixture. The paste was kept at a constant temperature (55°C). A small cotton swab (approximately 7×3 mm) was dipped into the liquified paste, then immediately rolled over the inoculated areas so as to deliver a thin, uniform film of the paste over the stem surface. Each inoculated area was thus covered with approximately 17 μg of the chemical. The number of lesions and brown streaks that developed was compared with the number that developed on comparable inoculated plants treated with lanolin only. This method has been used continuously for 2 years in selecting compounds that affect the multiplication of SBMV. Thiouracil and noformicin (MK-61), both known to possess antiviral activity (4, 5), reduced lesion production by approximately 70 and 95 per cent, respectively, when used repeatedly in the test described here. To test further the effectiveness of a compound, plants

FIG. 1. Method of inoculating Pinto bean hypocotyls with southern bean mosaic virus inoculum. Three drops (approximately 0.05 ml each) of inoculum are shown on the hypocotyl. The 2 uppermost drops contain Carborundum, and the stem surface covered by the center one is about to be sacrified with glass rod.

FIG. 2. Pinto bean hypocotyl, showing A) an inoculated area and B) an internal streak of dead tissue extending downward from it.

can be inoculated as previously described and the chemical applied to the uninjured hypocotyl a short distance below the inoculated areas. In this way the effectiveness of the compound in reducing or preventing movement of the virus through uninjured plant tissues can be evaluated.

Methods of tracing pathway of virus application.—Three methods were used to determine the pathway of virus movement in stems: 1) histological observations; 2) triphenyltetrazolium chloride color-reaction tests; and 3) return-inoculations with juice expressed from small stem segments or from dissected tissues.

The segments used for histological studies were collected and fixed in Navashin's solution at appropriate intervals following inoculation, dehydrated and embedded by the tertiary butyl alcohol method, sectioned into segments 12 μ thick, and stained with a modified triple stain (3).

For the tetrazolium test, thin (1–2 mm) slices of stems were placed immediately in a 1 per cent aqueous solution of the compound, and the rate at which red color developed in these slices was compared with that in comparable pieces from hypocotyls of virus-free plants of the same lot in the same stage of development.

For inoculation of indicator plants, segments or dissected tissues of infected hypocotyls were placed in amounts of water equal to about 25 or 50 times their weight and then were frozen. Later they were ground, and the extracted, diluted juice was used as inoculum. A possible limitation of this method is that the dilution of the juice might have been too great to give positive virus tests with tissues that contained very small amounts of the virus.

Entrance and transverse movement of virus.—For the inoculations to be successful, it was necessary that the virus inoculum be applied at the time of scarification. A parallel situation occurred with leaf inoculations (1). Scarification of the hypocotyls broke the outer walls of some epidermal cells and in some instances the walls of cortical cells immediately beneath the epidermis (Fig. 3, 4). Within 4 hours, injured cells formed "scabs" that effectively prevented infection when application of inoculum was delayed that long after scarification (Fig. 5, 6).

At the inoculated areas on the hypocotyls, no macroscopic or microscopic changes, except collapse and death of a few epidermal and adjacent outer cortical cells, were evident for about 48 hours following inoculation. These appeared to be the cells that had been most seriously damaged by scarification (Fig. 7, 8). At the end of 65 hours (Fig. 9) and 75 hours (Fig. 10), changes in epidermal and cortical cells directly inward from the region of inoculation were clearly visible: additional epidermal cells collapsed and died during this period, and the cortical cells beneath them shrank in size, their walls became wrinkled, and the cells showed evidences of necrosis as indicated by their staining reactions. Apparently the virus entered the cells of the pericycle, and perhaps the phloem, then traveled both up and down the stems in this region. It moved more quickly and to a greater distance downward than upward. The cells of the pericycle were killed rapidly following invasion by the virus, particularly if they were infected before they had become differentiated into mature fibers.

Ninety-six hours after inoculation, all of the cells of the epidermis, cortex, and endodermis directly beneath the area of inoculation were dead and nearly all were collapsed (Fig. 11). Cells of the pericycle inward from the inoculated area also were dead but not collapsed, and a few cells of the outer phloem were involved. A cross section of a scarified but noninoculated stem of the same age severed at the same level is shown for comparison (Fig. 12). Sections of stems at 144 hours (Fig. 13) and 240 hours (Fig. 14) after inoculation showed relatively little additional change in tissues directly inward from the inoculated areas, except further shrinkage of tissue and the involvement of cambium and xylem. This greater damage may have resulted in part from dessiccation owing to loss of protection previously provided by the killed tissues adjacent to the cambium and xylem. Plants allowed to grow for 20 days after inoculation showed compara-

TABLE 1.—*Extent of streaking and distance of downward movement of virus, as measured by 2 methods, in bean stems 4 days after inoculation with southern bean mosaic virus*

Plant number	Distance in mm downward from point of inoculation		
		Virus movement as shown by indicated test	
	Brown streak	Tetrazolium	Indicator plants
1	32	28	40
2	35	36	40
3	26	44	32
Average	31	36	37

tively little additional effects in tissues directly inward from the inoculated areas.

Longitudinal movement of virus.—One hundred twenty hours after inoculation of 1 group of plants, brown streaks were visible extending downward within the hypocotyls for a distance of approximately 25 mm from the site of inoculation (Fig. 2). To relate histological symptoms with the presence of the virus, stem portions of 2 of these plants were used for histological studies and the adjacent portions of the same stems for detection of the virus by color reaction with triphenyltetrazolium chloride. The stems were severed just above the soil line and, beginning at the inoculated areas, cut into 10-mm segments until the lower end of the visible streaking was reached. Freehand sections 1½–2 mm in thickness were cut from the basal ends of each segment and quickly placed in the 1 per cent aqueous tetrazolium solution. The remainder of each segment was fixed in Navashin's solution for histological study. Also, from the point at which visible streaking was evident in the hypocotyl, a single long segment, including the cotyledonary node and about 25 mm of the first internode, was fixed for histological study.

Injured and dead cells were present in greater numbers near the region of inoculation than at even relatively short distances above or below. The affected pericycle, endodermis, and phloem cells were directly in line with the inoculated areas for 35 mm (Fig. 15) and 25 mm (Fig. 16) below, and 15 mm (Fig. 17) above them. The tetrazolium tests indicated, however, the presence of virus or its effects for 40–50 mm below the points of inoculation. The visible streaking, as noted above, was evident for only 25 mm below the point of inoculation. Thus the tetrazolium tests indicated the presence of virus at least 5 mm below the histological effects and at least 15 mm below the visible streaking.

TABLE 2.—*Relative amounts of southern bean mosaic virus in various tissues of hypocotyls at and below inoculated areas as determined by inoculation of indicator plants*

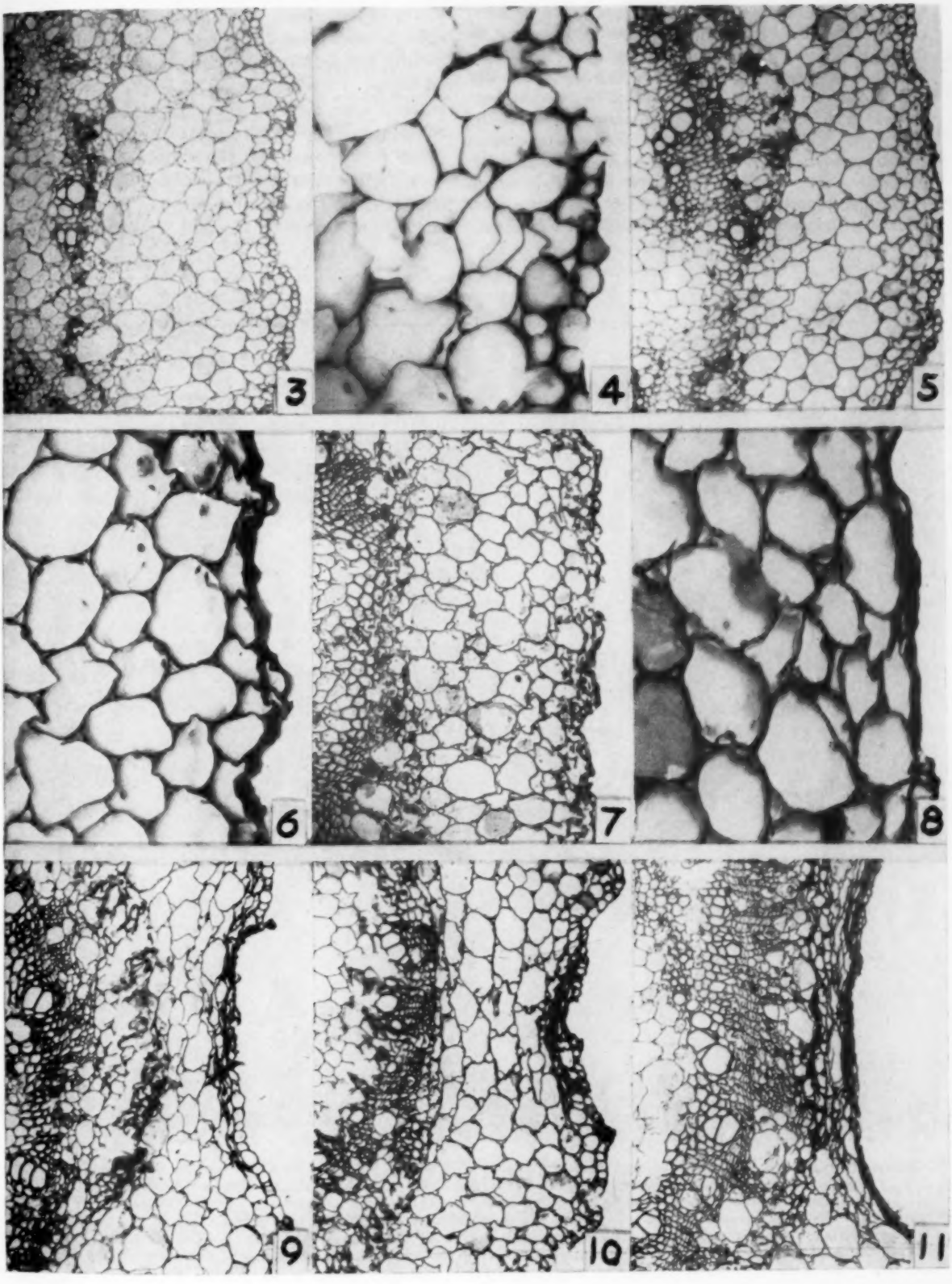
Description of hypocotyl segment	Tissues dissected	Relative amounts[c] of virus present at indicated interval after inoculation		
		1 Day	2 Days	6 Days
5-mm segment, including inoculated area[a]	Inoculated area and adjacent cortex and epidermis	None	High	High
	Remaining epidermis and cortex	None	Slight	Slight
	Pericycle and phloem	None	None	Slight
	Stele	None	Slight	Slight
5-mm segment, beginning 5 mm below bottom of first segment	Epidermis and cortex	None	None	None
	Pericycle and phloem	None	None	None
	Stele	None	None	None
	Brown streak in pericycle and phloem[b]			Slight

[a] See Figure 18.

[b] Brown streak dissected separately from the rest of the pericycle and phloem on the sixth day.

[c] Based on number of inoculated areas that became infected when inoculum from the dissected tissues was used on Pinto bean hypocotyls (67–100 per cent of inoculated areas infected = high; 33–66 per cent = moderate; 1–32 per cent = slight.

FIG. 3–11. Transections of Pinto bean stems inoculated with southern bean mosaic virus.—FIG. 3. Transection from hypocotyl fixed immediately after inoculation (scarification), showing injury to epidermal and cortical cells.—FIG. 4. Enlargement of portion of epidermis and cortex shown in Figure 3.—FIG. 5. Transection from hypocotyl inoculated 4 hours after scarification and then fixed immediately, showing "scab" composed of epidermal residue.—FIG. 6. Enlargement of portion of cortex and epidermal residue shown in Figure 5.—FIG. 7. Section from hypocotyl fixed 48 hours after scarification and inoculation.—FIG. 8. Enlargement of portion of cortex and epidermal residue shown in Figure 7.—FIG. 9. Transection of hypocotyl 65 hours after inoculation, showing considerable shrinkage and necrosis of epidermal and cortical cells.—FIG. 10. Transection 75 hours after inoculation, showing additional injury.—FIG. 11. Transection from hypocotyl fixed 96 hours after inoculation, showing marked shrinkage and necrosis in cells of the epidermis, cortex, endodermis, and pericycle.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Additional tests were made, therefore, to determine by the tetrazolium method and by inoculation of indicator plants the distance the virus moved downward in the hypocotyls. Three plants were inoculated, and 96 hours later the lengths of the brown streaks in the hypocotyls were measured and the hypocotyls sectioned into 4-mm segments beginning immediately below the inoculated areas. Alternate segments were frozen immediately to be used later as sources of inoculum. The remaining segments were sliced into 4 1-mm pieces and placed immediately in a 1 per cent solution of triphenyltetrazolium chloride for a comparison of their color changes with those in comparable pieces of hypocotyls of virus-free plants of the same age. In each case there was the possibility that a positive test for virus might have been obtained for an additional 4-mm section of hypocotyl had not that segment been used for the alternate test. The tetrazolium tests and results of inoculation of indicator plants both showed that the virus was present as far as or farther than the visible brown streaks extended in the hypocotyls of all 3 plants (Table 1). All 3 methods of following the path of movement of the

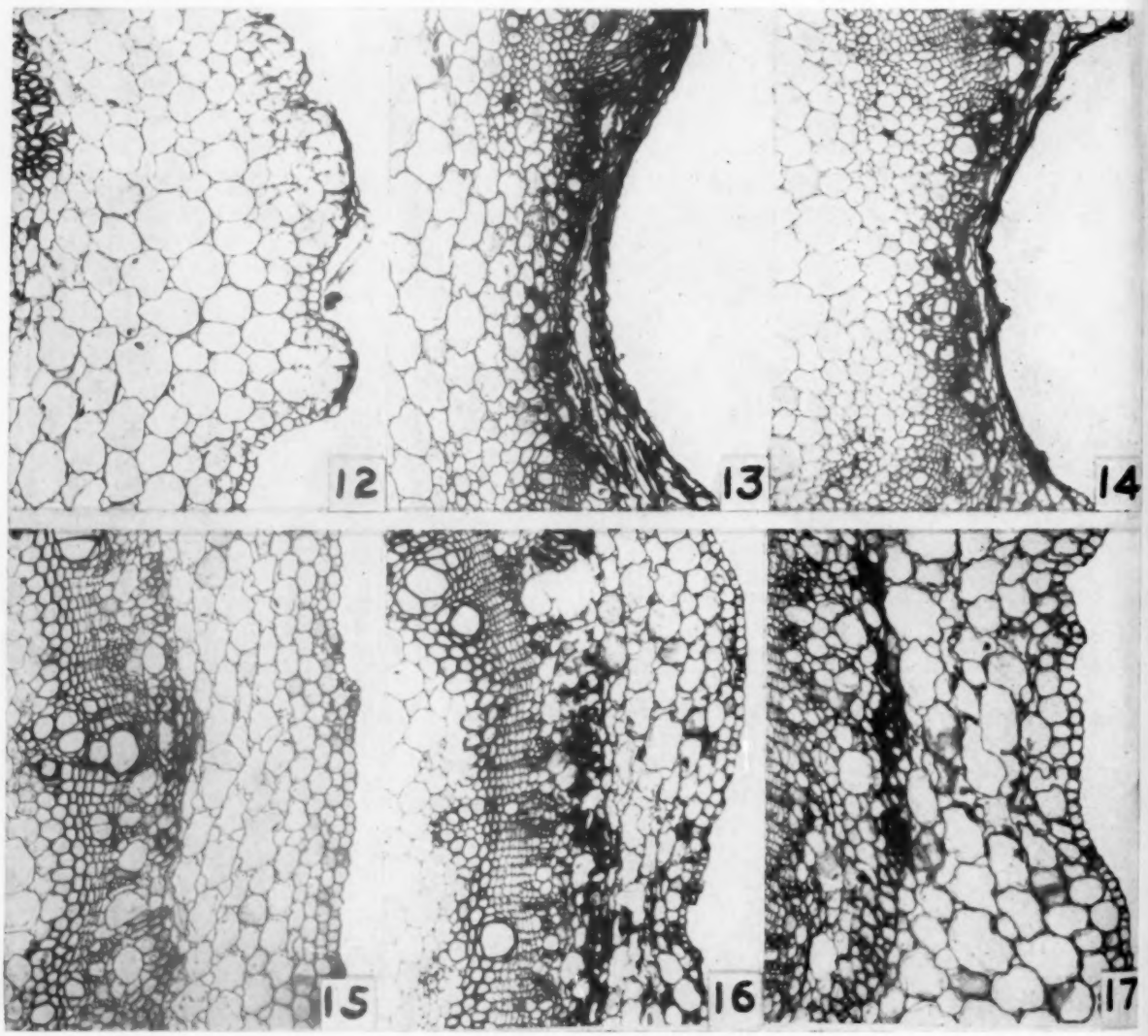

FIG. 12–17. FIG. 12. Scarified but noninoculated section of hypocotyl 96 hours after scarification, showing "scab" formation by dead epidermal cells and the beginning of a cork cambium beneath.—FIG. 13. Transection from hypocotyl fixed 144 hours after inoculation, showing more extensive injury than in Figure 11.—FIG. 14. Transection from hypocotyl fixed 240 hours after inoculation, showing little additional change over that in transection taken at 144 hours.—FIG. 15. Transection taken at 35 mm below point of inoculation, which is 10 mm below the end of visible streak.—FIG. 16. Transection taken at 25 mm below point of inoculation, showing lateral extension of effects of the virus in the endodermis and pericycle.—FIG. 17. Transection of first internode about 15 mm above point of inoculation, showing necrosis of only endodermis and pericycle.

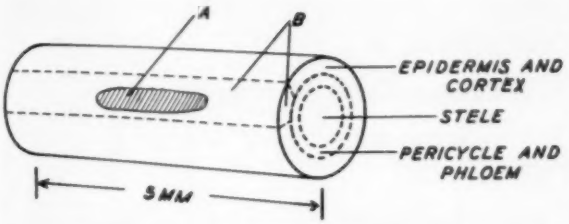

FIG. 18. Diagram of segment of Pinto bean hypocotyl, showing A) inoculated area, B) adjacent epidermis and cortex, and C) other tissue samples dissected for use in the inoculation of indicator plants for the detection of the presence of virus.

virus indicate presence of virus along the entire pathway.

The path of virus movement was traced in more detail by dissecting segments of infected hypocotyls and using these for inoculation of indicator plants. One area on each of 15 plants was inoculated. Hypocotyl segments from each of 5 plants were removed 1, 2, and 6 days after inoculation. Two 5-mm segments (one including the inoculated area and the other beginning 5-mm below the bottom of the first segment) were taken from each hypocotyl. Each segment was dissected to obtain tissue samples made up of the epidermis and cortex combined, of the pericycle and phloem combined, or of the stele (Fig. 18). The samples were obtained by carefully separating the tissues at the endodermis and cambium layers. No attempt was made, therefore, to account for the endodermis and cambium in the final samples. Care was taken to remove the inoculated portion of the epidermis together with adjacent epidermal and cortical tissues. The visible brown streaks in the pericycle also were separated from the rest of the parts. Sufficient water was added to each tissue sample to make a 1:25 dilution of the juice. Inoculations of indicator plants revealed that within 6 days after inoculation the virus had moved throughout the 5-mm segment of the hypocotyl that included the inoculated area and down the hypocotyl via the pericycle and phloem region. Movement was confined to cells in the brown streaks (Table 2).

The histological data, tetrazolium tests, and results of inoculation of indicator plants showed that movement of the virus occurred slowly from the epidermis or the outermost layer of cortical cells (where it was placed by inoculation) inward through the cortex into the inner tissues of the stem. During this inward movement, the virus progressed about 0.5–1 mm during the first 2 days. After the pericycle-phloem tissues had become infected, the virus moved downward at a greatly accelerated rate, i.e. 30–40 mm during the next 2 days.

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EFFECTS IN VITRO OF SOME ANTIBIOTICS ON THIRTY-TWO XANTHOMONAS SPECIES OCCURRING IN INDIA [1]

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SUMMARY

Five commercially available antibiotics, aureomycin, terramycin, sodium penicillin G, dihydrostreptomycin, and chloromycetin were tested *in vitro* against 32 *Xanthomonas* species occurring in India. The antibiotics were tested by cup-assay method in concentrations of 60 μg per ml for aureomycin, terramycin, and chloromycetin; 20 μg per ml for dihydrostreptomycin; and 50 units per ml for sodium penicillin G. Except penicillin, the antibiotics showed varying degrees of inhibition of growth against all the *Xanthomonas* species tested. The 32 Xanthomonas species were tested also with high concentrations of penicillin (100–1000 units per ml). At concentrations of 500 units and above, only 12 of the 32 species were sensitive. Cultures of *X. alfalfae* and of *X. cassiae* were mixed and then plated out on nutrient agar containing 1000 units of penicillin per ml. Host inoculation tests showed that all colonies that developed were *X. alfalfae*. The possibility of using this reaction for separating certain closely related species is discussed.

Literature on the action of antibiotics on phytopathogenic bacteria is relatively meager although considerable studies have been made on effects of antibiotics on bacterial pathogens of man and animals. Recent investigations by several workers have indicated the efficacy of antibiotics in controlling diseases incited by phytopathogenic bacteria (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15). Effects *in vitro* of some of the commercial antibiotics on bacterial plant pathogens have been reported from time to time, and some *Xanthomonas* species have been included in the list of bacteria tested. So far, no attempt has been made to study effects of antibiotics on 1 large group of related species of phytopathogenic bacteria or to study any underlying uniformity or differences in the reaction of members of a group to antibiotics. In the present paper, an account is presented of the effects of some of the commercially available antibiotics on 32 *Xanthomonas* species occurring in India.

MATERIALS AND METHODS.—In a wet tropical climate like that of India, the *Xanthomonas* species that incite leaf spots predominate in number. The 32 *Xanthomonas* species taken for study in the present paper are from culture collections maintained at the Plant Pathology Division, College of Agriculture, Poona, Bombay State.

In the preliminary tests, the appropriate concentrations of the antibiotics required for fairly measurable zones of inhibition were determined with Difco sensitivity discs: later, for actual tests, solutions of the antibiotics freshly prepared in phosphate buffer solutions (pH 7) were used. The different antibiotics tested were the following: sodium penicillin G, 50 units per ml; dihydrostreptomycin, 20 μg/ml; aureomycin (7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide), 60 μg/ml; terramycin (4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide) 60 μg/ml; and chloramphenicol (D(-)-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]acetamide), 60 μg/ml. The phosphate buffer alone served as control. The nutrient agar medium used for pouring plates had the following composition: beef extract, 3g; peptone (Difco), 10g; yeast extract (Difco), 5g; agar, 20g; and distilled water, 1 liter. The medium was adjusted to pH 7. Each species was grown at 30°C for 40 hours in a liquid medium prepared as given above but without the agar component. The growth of the bacteria in all cases was uniform and profuse. To each Petri dish (10 cm in diameter), 20 ml of the nutrient agar medium seeded with 1 ml of thoroughly mixed bacterial suspension was poured and cooled. The effect of the antibiotics was tested by the cup-assay method (13) in which porcelain fish spine beads were used. The Petri dishes were incubated at 5°C for 2 hours to permit the diffusion of the antibiotic in the agar, after which they were held at 30°C for 48 hours to permit growth of the test organism. The readings of the zones of inhibition were taken after 48 hours. All tests were carried out in triplicate.

RESULTS.—Cultures of *X. cassiae* and *X. alfalfae* were mixed and plated out on nutrient agar by the dilution-plate technique. Before the agar was poured, sterile sodium penicillin G solution in phosphate buffer was added to the plates in an amount sufficient to bring the final concentration of penicillin in the agar to 1000 units per ml. The agar at the time of pouring was as cool as possible, since heat quickly destroys penicillin in aqueous solution. After incubation for 3 days, the developing colonies were picked out at random, grown separately on agar slants, and used to inoculate plants of *Cassia tora* and of *Medicago sativa*. The 20 colonies that were thus isolated and tested caused infections in the latter but not in the former. This indicates that all were *X. alfalfae*,

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TABLE 1.—*Inhibition of Xanthomonas species by antibiotics*

Test organism	Host	Inhibition zone in mm[a]				
		Aureomycin	Terramycin	Penicillin G	Streptomycin	Chloramphenicol
X. alfalfae (Riker et al) Dows.	*Medicago sativa* L.	27	29	0	25	36
X. alysicarpi-vaginalis Patel et al	*Alysicarpus vaginalis* DC.	17	22	0	35	30
X. badrii Patel et al	*Xanthium strumarium* L.	26	29	0	28	37
X. betlicola Patel et al	*Piper betle* L.	30	33	0	25	42
X. bilvae Patel et al	*Aegle marmelos* Corr.	33	32	0	27	48
X. brideliae Bhatt & Patel	*Bridelia hamiltoniana* Wall.	16	22	0	24	27
X. buteae Bhatt & Patel	*Butea fondosa* König	30	32	0	27	42
X. cajanii Kulkarni et al	*Cajanus cajan* Millsp.	21	23	0	32	35
X. campestris (Pam.) Dows.	*Brassica oleracea* L.	30	32	0	22	42
X. cassiae Kulkarni et al	*Cassia tora* L.	28	32	0	27	42
X. citri (C. Hasse) Dows.	*Citrus aurantifolia* SW.	34	34	0	22	26
X. clerodendroni Patel et al	*Clerodendron phlomoides* L.	30	32	0	26	39
X. cyamopsidis Patel et al	*Cyamopsis tetragonoloba* (L.) Taub.	32	35	0	25	38
X. desmodii Uppal & Patel	*Desmodium diffusum* DC.	28	34	0	26	26
X. desmodii-gangetici Uppal et al	*Desmodium diffusum-gangeticum* DC.	22	29	0	29	35
X. erythrinae Patel et al	*Erythrina indica* Lam.	20	18	0	22	30
X. lawsoniae Patel et al	*Lawsonia alba* Lam.	31	33	0	25	40
X. malvacearum (E.L.Sm.) Dows.	*Gossypium hirsutum* L.	33	36	0	29	44
X. melhusii Patel et al	*Tectona grandis* L.	20	20	0	29	35
X. phaseoli-sojense (Hedges) Dows.	*Glycine max* L. (Merr.)	20	22	0	24	34
X. poinsetticola Patel et al	*Euphorbia pulcherrima* Willd.	29	33	0	26	38
X. ricinicola (Elliott) Dows.	*Ricinis communis* L.	32	34	0	26	42
X. rothiae Patel et al	*Rothia trifoliata* Pers.	21	23	0	26	30
X. sesbaniae Patel et al	*Sesbania aegyptiaca* Poir.	30	31	0	27	40
X. stizolobicola Patel et al	*Stizolobium deeringianum* Bort.	20	22	0	28	31
X. tamarindii Patel et al	*Tamarindus indica* L.	20	25	0	32	23
X. tephrosiae Bhatt & Patel	*Tephrosia purpurea* Pers.	17	24	0	29	28
X. trichodesmae Patel et al	*Trichodesma indicum* DC.	21	31	0	20	30
X. trilobii Patel et al	*Phaseolus trilobus* Ait.	20	23	0	29	35
X. uppali Patel	*Ipomoea muricata* R. & Sch.	18	22	0	27	34
X. vesicatoria (Doidge) Dows.	*Capsicum anuum* L.	29	29	0	27	39
X. vignicola Burk.	*Vigna sinensis* (L.) Endl.	28	34	0	26	36

[a] Average of 3 replicates.

and provides additional evidence that *X. cassiae* is inhibited completely by penicillin at high concentration of 1000 units.

The data of the first test (Table 1) indicate a very uniform type of reaction of the *Xamthomonas* species towards a given antibiotic. All the species were sensitive to aureomycin, terramycin, chloromycetin, and dihydrostreptomycin in the concentrations used. None of them are sensitive to penicillin at the concentration of 50 units per ml. This is understandable in view of the fact that all *Xanthomonas* species are Gram-negative. Reports by other investigators (2, 9) of slight inhibition of *Xanthomonas* species by penicillin at concentration of 10 units per ml are of questionable significance; the diameter of the zone of inhibition was given as being less than 10 mm, whereas the diameter of the cup used in the cylinder-plate assay itself must have been 5–8 mm.

In the second series of experiments, the effects of higher concentrations of penicillin on different *Xanthomonas* species were tested. Penicillin (sodium penicillin G) concentrations of 100 units, 250 units, 500 units, and 1000 units per ml were prepared in phosphate buffer solution of pH 7 and tested by the cup-assay method against the *Xanthomonas* species. Results indicated that the 32 *Xanthomonas* species fall into 2 groups, one sensitive to penicillin at concentrations of 500 units and above and the other insensitive even up to concentrations of 1000 units per ml (Table 2). Among the species that were sensitive to penicillin at high concentrations, *X. malvacearum* was the most susceptible and *X. alsycarpi-vaginalis* most resistant.

TABLE 2.—*Inhibition of some Xanthomonas species by high concentrations of penicillin*

Test organism[a]	Inhibition zone in mm at indicated concentrations (μ/ml)			
	100	250	500	1000
X. alysicarpi-vaginalis	0	0	0	13
X. bilvae	0	12	16	24
X. cajanii	0	0	11	19
X. cassiae	0	0	15	26
X. desmodii-gangetici	0	14	16	20
X. erythrinae	0	11	11	20
X. malvacearum	0	16	20	32
X. melhusii	0	0	12	14
X. poinsetticola	0	0	11	20
X. stizolobicola	0	11	14	21
X. uppali	0	11	12	15
X. vesicatoria	0	14	14	24

[a] Species not listed (of those listed in Table 1) were not affected by penicillin at the concentration used.

DISCUSSION.—The finding that some *Xanthomonas* species are sensitive to high concentrations of penicillin provides a very important biochemical character for the differentiation of *Xanthomonas* species. Several investigators have pointed out that most of the *Xanthomonas* species show the same range of morphological and biochemical characters, so that the differentiation of species has been based chiefly on host specificity. Even serological data have not been of much value in separating the species. In some cases, further difficulty arises from the fact that a single *Xanthomonas* species may parasitise more than 1 host. In such cases, any differences in the biochemical reaction would be of great value. In the present case, 2 instances were noted where sensitivity to high concentrations of penicillin could afford a means of differentiating the species.

Xanthomonas citri and *X. bilvae* are 2 closely related species parasitising citrus and *Aegle marmelos*, respectively, both hosts being members of the *Rutaceae*. *X. citri* will infect *A. marmelos* but *X. bilvae* does not infect citrus plants. In the present study, it was shown that *X. bilvae* is sensitive to penicillin even at 250 units per ml, whereas *X. citri* is insensitive, even at 1000 units per ml and above.

Another instance is that of *X. cassiae* and *X. alfalfae*. Although neither will infect the usual host of the other, they have a common host in *Pisum sativum*. On *P. sativum*, the 2 species are indistinguishable by the morphological and biochemical characters known at present. In the present studies, *X. cassiae* was sensitive to penicillin at concentrations of 500 μ/ml and above, whereas *X. alfalfae* was insensitive.

The present findings suggest that it might be desirable to include the reaction to penicillin at a concentration of 1000 units per ml as one of the characteristics used in describing *Xanthomonas* species; however, the work of English et al (6) shows that resistant strains may develop as a result of continued use of antibiotics. If this should occur frequently, characterization of species based on sensitivity to penicillin would be of little value, particularly in the case of pathogens whose control is attempted by use of antibiotics.

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A STUDY OF MELON AND SQUASH MOSAIC VIRUSES[1]

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SUMMARY

Thirteen viruses that were infective on cucurbits but not on tobacco were obtained from Wisconsin, California, and Florida. On the basis of physical properties and insect transmission, they were divided into 2 groups: the melon mosaic group and the squash mosaic group. Viruses in the melon group were transmitted by peach and melon aphids; activity was lost in 10 minutes at 60°C, during 28 days aging *in vitro* (with 1 exception), and at a 5×10^{-3} dilution. Viruses in the squash group were not transmitted by aphids, and were not inactivated in 10 minutes at 60°, during 28 days aging *in vitro*, or at 10^{-4} or 10^{-5} dilution. Purified preparations of the viruses in the squash mosaic group in all cases showed, by electron microscopy, spherical or polyhedral particles. The diameter of particles of 1 isolate averaged 367A. Purification and electron microscopy of viruses in the melon group were not successful.

The melon mosaic group contained a Wisconsin isolate obtained from infected muskmelon seed, Freitag's cantaloupe mosaic virus collected in California, an isolate from infected squash collected in Florida, and Anderson's watermelon and yellow watermelon mosaic viruses collected in Florida. All were infectious on watermelon. They are regarded as strains of the same virus, which is designated as melon mosaic virus.

The squash mosaic group contained Freitag's squash mosaic virus from California, 2 Wisconsin isolates from wild cucumber, Anderson's typical and latent muskmelon viruses from Florida, Freitag's cucurbit ring virus from California, and Freitag's wild cucumber mosaic virus from the same state. All but the last of these failed to infect watermelon and all but the last had similar antigenic properties. They are regarded as strains of squash mosaic virus. Freitag's wild cucumber mosaic virus differs from other members of the squash mosaic group in that it infects watermelon systemically, and it is serologically distinct from other members of the group. It is regarded, therefore, as a distinct virus and it is designated as wild cucumber mosaic virus.

Several distinct viruses are known to infect cucumber (*Cucumis sativus* L.) and various other cucurbits. The most common of these is cucumber mosaic virus (CMV). Numerous distinct strains have been described and placed within CMV on the basis of cross protection in zinnia (*Zinnia elegans* Jacq.) with Price's local-lesion strain (11). Cucumber virus 3 (CV3) and cucumber virus 4 (CV4) differ markedly from CMV in physical properties: they infect only cucurbits (1), and they are serologically related to tobacco mosaic virus (5). They have not been reported in the United States. Tobacco ring spot virus (TRSV), which also infects cucurbits, has a very wide host range and is distinct in properties from CMV, CV3, and CV4. A seed-borne virus of muskmelon observed by Kendrick (9) was studied later by Freitag (6) and designated as squash mosaic virus in 1941. In 1952, Freitag (7) stated that he was able to differentiate 7 distinct cucurbit viruses in California on the basis of symptoms, insect vectors, and physical properties, although the details of such distinctions were not presented. These 7 viruses were designated as cucumber mosaic, squash mosaic, muskmelon mosaic (cucurbit ring mosaic), wild cucumber mosaic, cantaloupe mosaic, western muskmelon mosaic, and muskmelon vein necrosis viruses. All except cucumber mosaic virus had limited host ranges. In 1954, Anderson (2, 3) in Florida described watermelon mosaic virus and yellow watermelon mosaic virus from *Cucurbita pepo* var. *melo-pepo* (L.) Alef., muskmelon mosaic virus from *Cucumis melo* L., and latent muskmelon mosaic virus from *Melothria pendula* L. The 4 viruses just mentioned were not transmitted to plants outside the cucurbits.

In the present investigation, a critical comparison has been made of a number of cucurbit virus isolates from Wisconsin, California, and Florida. These viruses, except for a typical strain of CMV, were not infectious on tobacco, as is TRSV, and their physical properties were distinct from those of CV3 and CV4.

LIST OF VIRUS ISOLATES STUDIED.—The cucurbit virus isolates studied, except CMV, are listed below in 2 groups, the squash mosaic group and the melon mosaic group. The data that establish the identity of each group are presented later in the text. Each virus isolate is given a symbol. The last 2 letters of the symbol of each isolate in the squash mosaic group are SV; for those in the melon mosaic group they are MV. The first letter (and in 1 case the first 2 letters) in the symbol was chosen at random to distinguish one isolate from another within a given group.

Squash mosaic group.—WSV, "squash mosaic virus" received from J. H. Freitag in California in 1947; FSV, "squash mosaic virus" received from the same source as WSV in 1953; RSV, "cucurbit ring virus" received from the same source as WSV in 1953; WCSV, "wild cucumber mosaic virus" received from the same source as WSV in 1953; SSV, "severe squash mosaic virus" isolated from wild cucumber in Wisconsin in 1953; MSV, "mild squash mosaic virus" isolated from wild cucumber in Wisconsin in 1953; OSV, "typical muskmelon mosaic virus" received from C. W. Anderson in Florida in 1955; LSV, "latent musk-

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melon mosaic virus" received from the same source as OSV in 1955.

Melon mosaic group.—RMV, a seed-borne virus isolated in 1949 at Madison, Wisconsin, from diseased muskmelon plants; FMV, a virus collected from diseased squash plants in southern Florida in 1952; WMV, "cantaloupe mosaic virus" received from J. H. Freitag in California in 1953; AMV, "watermelon mosaic virus" received from C. W. Anderson in Florida in 1954; YMV, "yellow watermelon mosaic virus" received from the same source as AMV in 1954.

The strain of CMV used was isolated from spinach in southeastern Wisconsin in 1941. It is similar to the usual wild type isolated from cucumber in Wisconsin. Price's No. 6 local-lesion indicator strain (11) was used to determine whether or not CMV was present in certain collections.

EXPERIMENTAL RESULTS.—*Occurrence of viruses in wild cucumber in Wisconsin.*—Since certain of the viruses used are seed borne, it became of interest to determine whether any such viruses were endemic in wild cucumber in Wisconsin. In 1953, collections of leaves from plants affected with mosaic were made in 6 areas in central and southern Wisconsin. Extracts from each collection were inoculated mechanically to cucumber (var. National Pickling) and to tobacco (*Nicotiana tabacum* L. var. Havana 38). Cucumber was infected in all cases, but in many instances tobacco was not. Cowpea (*Vigna sinensis* (L.) Endl. var. Black) and zinnia were inoculated with extracts from those tobacco plants that did become infected. In all cases, local lesions on cowpea and systemic mottle on zinnia resulted. The infected zinnia plants were later inoculated with Price's No. 6 local-lesion indicator strain of CMV. No lesions developed. These results were considered conclusive evidence that the isolates that infected tobacco were CMV. Those isolates infectious on cucumber but not on tobacco were studied further on several cucurbit hosts. Two types of symptoms were observed on pumpkin (*Cucurbita pepo* L. var. Small Sugar) and on squash (*C. pepo* L. var. Zucchini). One lot of the viruses produced a distinct mottle, the other produced a mild mottle. A representative of each lot was selected and designated as SSV and MSV, respectively, and added to the list of viruses used for further study. As will be shown later, both of these isolates resembled closely the squash mosaic virus of Freitag.

Seed transmission in wild cucumber.—Seeds were selected from infected vines of wild cucumber from 7 locations in Wisconsin. The seeds were stratified in moist sand and allowed to freeze during the ensuing winter. They were then germinated, set in individual pots, and allowed to grow until symptoms on some plants were well developed. Each plant showing symptoms was assayed by inoculation to tobacco and to cucumber. It was soon obvious that an SSV-type virus had been present in some of the wild cucumber seeds and that a CMV-type virus had been present in others. On cucumber, the SSV-type virus induced vein clearing and no distortion of the leaves in the early stages, whereas those infected with CMV showed chlorosis, epinasty, and stunting. Moreover, those isolates that showed symptoms of infection by CMV on cucumber were infectious on tobacco, whereas those that showed mild vein clearing were not. Both viruses were recovered from seed from 5 localities, only SSV from 1 locality, and neither virus from 1 locality. Of the total number of seed tested, approximately 2 per cent carried one or the other virus. It should be pointed out that although MSV was found about as frequently as SSV in wild cucumber in the 1953 survey, no recovery of the former was obtained through wild cucumber seed. No explanation of its means of overwintering in Wisconsin is known.

Host ranges of the viruses.—The species of plants to be tested were grown in soil in 4-in. pots in the greenhouse. Five young plants of each lot were inoculated mechanically on the cotyledons or first true leaves, by the aid of Carborundum, with infectious crude sap from diseased pumpkin (var. Small Sugar). Fifteen to 20 days after inoculation, leaves were taken from above the point of inoculation and ground; the juice was extracted by squeezing through cheesecloth. The sap was inoculated mechanically to cucumber (var. National Pickling), except that *Cucurbita pepo* L. var. Uconn was inoculated in the case of WCSV since this isolate did not infect cucumber.

Only plants belonging to the family Cucurbitaceae became systemically infected with each virus except CMV (Table 1). The latter not only infected systemically all cucurbits tested except watermelon, but also *Nicotiana tabacum*, *N. glutinosa* L., *N. rustica* L., *Datura stramonium* L., and *Zinnia elegans*. It induced a hypersensitive reaction on watermelon.

All isolates in the squash mosaic group except WCSV infected all cucurbits tested except watermelon. This is in accord with the results of Freitag (8), who also reported his squash mosaic virus to be infectious on *Phacelia campanularia* Gray, *Lathyrus octoratus* L., *Pisum sativum* L., *Coriandrum sativum* L., and *Anthrisus cerefolium* Hoffm. The results with LSV are not in accord with those of Anderson (3), who reported that this virus did not infect cucumber and muskmelon. In our experiments, LSV induced very mild symptoms on these 2 hosts.

WCSV in this test induced only a hypersensitive reaction on watermelon. In a subsequent test on 15 varieties of watermelon, systemic mottle appeared on many plants. This isolate was not transmitted to cucumber, *Cucurbita moschata*, or *C. maxima* in this test. In a subsequent test positive results were secured with *C. maxima*.

The isolates of the melon mosaic group were infectious on all cucurbits tested.

Symptoms induced by the viruses.—With the exception of WCSV, all of the isolates in the squash mosaic group produced similar effects on cucumber, the major differences among isolates being in intensity of symptoms induced. Small chlorotic spots sometimes formed

TABLE 1.—*Reaction*[a] *of various hosts to inoculation with each of 14 cucurbit viruses*

Hosts	Squash mosaic group								Melon mosaic group					
	SSV	MSV	FSV	WSV	RSV	WCSV	OSV	LSV	RMV	WMV	FMV	AMV	YMV	CMV
Citrullus vulgaris	—	—	—	—	—	H[b]	—	—	+	+	+	+	+	H
Cucurbita moschata	+	+	+	+	+	—[c]	+	+	+	+	+	+	+	+
C. maxima	+	+	+	+	+	—[c]	+	+	+	+	+	+	+	+
C. pepo	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cucumis sativus	+	+	+	+	+	—	+	+	+	+	+	+	+	+
C. melo	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nicotiana tabacum	—	—	—	—	—	—	—	—	—	—	—	—	—	+
N. glutinosa	—	—	—	—	—	—	—	—	—	—	—	—	—	+
N. rustica	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Datura stramonium	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Vigna sinensis	—	—	—	—	—	—	—	—	—	—	—	—	—	H
Zinnia elegans	—	—	—	—	—	—	—	—	—	—	—	—	—	+

[a] H indicates a hypersensitive reaction following inoculation; + indicates positive infection; — indicates no symptoms and no recovery from inoculated plants. Each value is the result of a test with 5 plants.
[b] In a subsequent test with WCSV on watermelon, systemic mottle occurred.
[c] In a subsequent test with WCSV on *Cucurbita maxima*, systemic mottle occurred.

on the inoculated cotyledons, and yellow areas developed along the veins of the first true leaf about 5 days after inoculation. A few yellow areas developed on subsequent leaves.

The viruses of the melon mosaic group produced symptoms much more slowly on cucumber than did those of the squash mosaic group. About 10 days after inoculation, a diffuse chlorosis developed in the first true leaf. The veins of the next leaf showed vein-clearing, and as the leaf continued to develop a yellow mottle appeared. There was no apparent recovery from symptoms as was found with the viruses of the squash mosaic group.

Cucumber plants infected with a virus of either group were only slightly stunted. On the other hand, cucumber plants infected with CMV showed marked stunting, and the leaves were severely mottled.

Although the viruses could be placed in 3 well-defined groups on the basis of symptoms in cucumber, no such grouping of the viruses could be made from the symptoms produced on the 3 species of *Cucurbita* used in the host-range study. CMV had the most severe effect. Yellow mottle appeared on the first and subsequent leaves, and the entire plants were severely stunted. On plants inoculated with any of the viruses of the squash mosaic group, with the exception of WCSV and MSV, the second or third true leaf became mottled about 7 days after inoculation. When the fourth or fifth leaf had developed, differences among certain of the viruses became clearly discernible. Plants infected with SSV were mottled, and as they continued to develop the leaves became more distorted in shape and often assumed a shoestring-like appearance. With FSV and WSV, distortion of the leaves was less pronounced. With RSV, OSV, and LSV, chlorotic rings appeared on the first mottled leaf as it expanded, and although fewer rings developed on subsequent leaves there was little or no distortion. On *Curcurbita pepo*, WCSV caused a yellow mottle unlike symptoms produced by any of the other viruses in the squash mosaic group. These yellow areas became less conspicuous as leaves expanded; there was no distortion. The first true leaf of plants inoculated with MSV showed necrosis, which was sometimes confined to the margin of the leaf but which often involved the entire leaf and the petiole. Symptoms were almost entirely masked in the next 3 or 4 leaves, but the newer leaves on older plants always became slightly mottled. Stunting of the plants was slight with each of the viruses in the squash mosaic group.

The most characteristic symptoms on species of *Cucurbita* incited by viruses in the melon mosaic group were observed also in the 4- or 5-leaf stage. RMV and WMV produced quite similar mild mosaic symptoms. The symptoms produced by FMV and by AMV were identical and differed from those produced by RMV and by WMV in that the mottled areas of a diseased leaf were greenish yellow in color and contained small dark green islands, which often were raised such that the leaves had a distorted appearance. The mottled areas produced by YMV had a conspicuous yellow color, similar to that of the mottle produced by WCSV of the squash mosaic group on *C. pepo*, but distinct from that of the mottle produced by other members of the melon mosaic group. In older plants, however, the leaves of plants infected with YMV became misshapen, a characteristic never observed with WCSV. Stunting was slight with all viruses in the melon mosaic group. Although certain features of the disease syndrome on squash and pumpkin were distinctive for one or another virus in either the squash mosaic or the melon mosaic group, these characteristics were not unique and thus could not be used as an easy and reliable means of identification.

Several varieties of watermelon were inoculated with each of the 14 cucurbit viruses. A given virus produced similar symptoms on the various varieties. Each of the viruses of the melon mosaic group produced mottle symptoms. WCSV induced large, spreading, necrotic spots on the inoculated cotyledons and small necrotic spots on the first true leaf. In some experiments, the plants were severely stunted or killed,

TABLE 2.—*Relative infectivities of extracts obtained from plants infected with each of 14 cucurbit virus isolates and heated for 10 minutes at various temperatures*

Virus group	Virus	Percentage of diseased test plants following inoculation with extracts heated at temperature (°C) indicated[a]				
		Control	50°	60°	70°	80°
Squash	SSV	100	100	80	0	0
	MSV	100	100	100	0	0
	FSV	100	100	60	0	0
	WSV	100	100	75	0	0
	RSV	95	90	45	0	0
	WCSV	100	90	60	0	0
	OSV	100	100	100	0	0
	LSV	100	95	95	0	0
Melon	RMV	100	80	0	0	0
	WMV	100	100	0	0	0
	AMV	95	5	0	0	0
	FMV	100	20	0	0	0
	YMV	100	0	0	0	0
Cucumber	CMV	100	100	30	0	0

[a] Ten plants were inoculated in each treatment in each experiment; the figures are averages of 2 or more experiments.

whereas in other tests a mild systemic mottle appeared. The remaining members of the squash mosaic group failed to infect watermelon, and these viruses were not reisolated from the inoculated plants. Anderson (3), however, reported local lesions in watermelon with OSV. A type of hypersensitivity similar to that which sometimes appeared with WCSV developed in watermelon inoculated with CMV. The virus became established only when the plants were held at 24° and 28°C, whereas at 16° no infection resulted.

Physical properties.—The resistance of the 14 viruses to exposure at various temperatures for 10 minutes, to aging *in vitro* at room temperature, and to dilution was studied by the usual methods. *Cucurbita pepo* L. (var. Small Sugar) was the source plant for each virus except CMV, for which *Nicotiana glutinosa* was used.

All viruses in the squash mosaic group were active after heating at 60°C but not at 70° (Table 2). Freitag (8) reported his squash mosaic virus isolate active at 70° but not at 80°. All were active at a dilution of 10^{-4}, but not all at one of 10^{-5} (Table 3). SSV, WSV, and FSV were inactive at a dilution of 10^{-6}. Freitag (8) reported slight activity of his isolate at 10^{-6}. All were active after standing *in vitro* at room temperature for 28 days (Table 4). Freitag (8) reported his isolate active after 6 weeks but not after 2 months.

The viruses in the melon mosaic group, by way of comparison, were inactivated by heating at 60°C (Table 2); they were inactive at a dilution of 5×10^{-3}, except for RMV (Table 3); and they were inactive after aging at room temperature *in vitro* for 28 days, except for WMV (Table 4). The muskmelon mosaic viruses reported by Rader et al (12) and by Aycock (4) resembled in physical properties those reported here in the melon mosaic group, whereas the properties reported by Porter and McWhorter (10) for a virus from wild cucumber in Oregon resembled more closely those of members of the squash mosaic group.

The properties of CMV in these tests conformed closely with those of the viruses in the melon mosaic group. It is to be noted that CV3 and CV4 withstand a temperature of 80°C, and remain active *in vitro* for 9 months or longer (1).

Insect transmission.—Aphid transmission tests were made with all 14 virus isolates. Virus-free adults of *Myzus persicae* Sulz. and *Aphis gossypii* Glover that had been starved 1 hour were used. Small Sugar pumpkin was used as the test plant. The results (Table 5) show that all isolates of the melon mosaic group as well as CMV were transmitted readily by each species of aphid, whereas no transmission of members of the squash mosaic group was secured. This is in accord with the report of Anderson (2, 3) on AMV, YMV, OSV, and LSV, and that of Freitag (7) on FSV, RSV, WCSV, and WMV.

Virus-free adults of spotted cucumber beetle (*Acalym-*

TABLE 3.—*Relative infectivities of various dilutions of extracts from plants infected by each of 14 cucurbit virus isolates*

Virus group	Virus	Percentage of diseased test plants following inoculation with dilution indicated[a]							
		Undiluted	10^{-1}	10^{-2}	10^{-3}	$5 \cdot 10^{-3}$	10^{-4}	10^{-5}	10^{-6}
Squash	SSV	100	100	100	100		90	50	0
	MSV	95	100	100	100		70	0	0
	WSV	100	95	85	95		90	40	0
	FSV	90	100	90	80		100	20	0
	RSV	95	90	95	65		45	0	0
	WCSV	100	100	95	90		35	0	0
	OSV	100	100	100	90		60	0	0
	LSV	100	90	95	70		50	0	0
Melon	RMV	95	90	85	70	5	0		
	WMV	100	100	85	40	0	0		
	FMV	90	90	90	60	0	0		
	AMV	95	95	80	35	0	0		
	YMV	90	90	70	20	0	0		
Cucumber	CMV	100	100	80	60	0	0		

[a] Ten plants were inoculated in each treatment in each experiment; the figures are the averages of 2 experiments.

TABLE 4.—*Relative infectivities of extracts obtained from plants infected with each of 14 cucurbit virus isolates and aged at room temperature for various periods*

Virus group	Virus	Percentage of diseased plants following inoculation with extracts aged for period indicated[a]				
		1 Day	2 Days	7 Days	14 Days	28 Days
Squash	SSV	100	100	100	100	100
	MSV	100	100	100	100	95
	WSV	100	100	95	100	100
	FSV	100	95	100	100	100
	RSV	100	95	100	100	60
	WCSV	100	100	100	100	70
	OSV	100	100	100	100	60
	LSV	100	90	95	100	50
Melon	RMV	100	90	65	40	0
	WMV	85	90	50	10	5
	FMV	95	90	50	20	0
	AMV	100	40	45	10	0
	YMV	100	70	50	30	0
Cucumber	CMV	95	90	60	20	0

[a] Ten plants were inoculated in each treatment; the figures are averages of 2 experiments.

TABLE 5.—*Aphid transmission tests with 14 cucurbit virus isolates*

Virus group	Virus	Percentage of test plants that became infected following attempted transmission by means of aphid species indicated[a]	
		Myzus persicae	*Aphis gossypii*
Squash	SSV	0	0
	MSV	0	0
	FSV	0	0
	WSV	0	0
	RSV	0	0
	WCSV	0	0
	OSV	0	0
	LSV	0	0
Melon	RMV	89	80
	WMV	79	90
	FMV	100	94
	AMV	68	79
	YMV	68	68
Cucumber	CMV	79	80

[a] Ten aphids were placed on each of 10 plants in each test; the figures are averages of 2 experiments or more.

ma duodecimpuncata Oliver) were used in transmission tests with SSV of the squash mosaic group and with RMV and AMV of the melon mosaic group. Forty Small Sugar pumpkin plants were used in each test. Twenty per cent of the plants became infected with SSV and none were infected with RMV or AMV. Freitag (7) has shown that FSV, RSV, and WCSV of the squash mosaic group are beetle-borne whereas WMV of the melon mosaic group is not. Insofar as tests have been made, then, the members of the squash group are beetle-borne and not aphid-borne whereas the reverse is true with members of the melon mosaic group.

Electron microscopy.—Small Sugar pumpkin plants were inoculated in the cotyledonary stage with each of the 8 isolates in the squash mosaic group. Symptoms appeared in 7 or 8 days and leaves were harvested at 3 weeks. Leaves were frozen and the juice was extracted by grinding the tissue in an Enterprise vegetable juicer. Ice cubes made from distilled water were added occasionally to the material to keep the system cold. Healthy control plants were grown along with the inoculated plants, and juice was extracted from them in the same manner. The extracted sap was clarified by centrifugation at 2000 RPM for 15 minutes in an SB-type International centrifuge. The particulate material that accumulated was discarded, and the supernatant was adjusted to pH 5 with 10 per cent acetic acid. The change in pH resulted in a change in color from dark to light green. The sap was centrifuged again at 2000 RPM for 15 minutes. The green sediment was discarded and the brown supernatant was centrifuged in a No. 30 rotor of a model L Spinco ultracentrifuge at 10,000 RPM for 10 minutes. The brown pellet was discarded, and the supernatant was centrifuged at 28,000 RPM for 90 minutes. The yellow pellet from the last centrifugation was suspended in 5 ml of distilled water. The suspensions from several centrifuge tubes were combined and centrifuged at 28,000 RPM for 90 minutes. The pellet, which appeared to be uniformly yellow in color except for a slight mixture of brownish material, was suspended in distilled water, and the suspension was centrifuged at 10,000 RPM for 10 minutes. The final pellet from infected material was almost free from brownish material. It was sticky in consistency and was suspended in distilled water only after vigorous rubbing with a "rubber policeman." The final pellet from extracts of healthy plants was much smaller than that from infected juice. It contained a comparable amount of brownish material, but it lacked the sticky yellowish sediment of the pellet from juice of infected plants.

The final pellets were suspended in 5 ml of distilled water. The suspensions of the virus preparations were used to inoculate pumpkin plants and were in the case of each isolate found to be infectious. They were diluted for the electron microscope mounts in 100 or 500 parts of water, depending on the yield of the isolate concerned. The preparation from healthy plants was diluted in 10 parts of water.

Electron microscope screens (200-mesh nickel lektromesh) were coated with a 0.2 per cent formvar solution in ethylene dichloride. After the screens were dried over calcium chloride, a small drop of virus or of the preparation from healthy plants was placed on each screen. When dry, the preparations were shadowed with uranium. Electron micrographs were taken on a model ENU-2b RCA electron microscope. The micrographs showed spherical particles of approximately the same size range for each virus preparation. This is in accord with the report of Takahashi and Rawlins on squash mosaic virus (15). The average diameter of

all particles measured in the SSV preparation was 367 A. Spherical particles were not present in preparations from extract from healthy plants. When Stahmann and Kaesberg (14) examined preparations of SSV made by the freeze-drying technique, they found the particles to be polyhedral rather than spherical in outline. Further studies on the physico-chemical properties of SSV are published elsewhere (13).

The same purification technique was applied to the isolates in the melon mosaic group, but infectivitiy was lost and no satisfactory preparations for electron microscopy were obtained.

Serological studies.—The isolates of the squash mosaic group were purified by the method just described. The purified preparations of SSV, WSV, FSV, and WCSV were injected intravenously into rabbits. A blood probe of about 15 ml was taken 1 week prior to the first injection to obtain normal serum for use as a control. Five 1-ml injections at 3-day intervals resulted in a titer of 1:32 with each virus isolate used. A 2-fold serial dilution of antiserum was made, and 0.3 ml of each dilution was added to an equal volume of a constant dilution of antigen. Physiologic saline solution was used as the diluent. Precipitin tests were made in 75 × 8-mm tubes in a water bath at 37°C.

Precipitin tests were conducted with each isolate of the squash mosaic group against each of the 4 antisera produced (Table 6). Homologous tests with each antiserum tested against purified virus as the antigen resulted in a pronounced precipitate up to 1:32 dilution. SSV, WSV, and FSV reacted to form a precipitate when added to the antiserum of any one of the 3 isolates; however, when added to WCSV antiserum neither of them formed a precipitate, nor did the antigen of WCSV when reciprocal tests were made with the heterologous antisera of SSV, WSV, or FSV. Additional precipitin tests were made with RSV, MSV, OSV, and LSV, and each formed a precipitate of SSV, WSV or FSV antisera up to 1:32 dilution, but they gave no precipitate in the presence of WCSV antiserum. When expressed sap cleared with acetic acid at pH 5.2 was used as the antigen, similar results were secured except that the reaction time for precipitin formation was longer than with corresponding dilutions of antiserum and purified virus.

No precipitate was formed when SSV or WCSV antiserum was added to acid-clarified extracts from plants infected by AMV, FMV, RMV, or CMV, an indication that CMV and members of the melon mosaic group were not serologically related to isolates of the squash mosaic group.

TABLE 6.—*Homologous and heterologous precipitin tests with antigens and antisera of virus isolates of the squash mosaic group*

Antigen	Reaction at 1-32 dilution of indicated antiserum[a]				
	SSV	WSV	FSV	WCSV	Normal serum[a]
SSV	+	+	+	—	—
WSV	+	+	+	—	—
FSV	+	+	+	—	—
MSV	+	+	+	—	
RSV	+	+	+	—	
LSV	+	+	+	—	
OSV	+	+	+	—	
WCSV	—	—	—	+	—
Healthy-plant extract[b]	—	—	—	—	—

[a] (+) Indicates a positive precipitin reaction, (—) indicates no precipitate formed.

[b] The antigen used here was a sap extract from healthy pumpkin plants treated in the same way as were sap extracts from diseased plants.

DISCUSSION.—Certain of the viruses conform closely to the authentic strain of squash mosaic virus received from California. Isolates that are sufficiently similar in host range, physical properties, insect transmission, and serological activity to be considered strains of squash mosaic virus (SMV) include 2 isolates from Wisconsin (SSV and MSV), the typical and latent muskmelon mosaic viruses received from Florida (OSV and LSV), the cucurbit ring virus from California (RSV), and 2 isolates of squash mosaic virus from California (WSV and FSV). Although another virus, wild cucumber mosaic virus (WCSV), is similar in physical properties to squash mosaic virus, it is regarded as a distinct virus largely because it differs serologically from squash mosaic virus. It also differs in host range from all others studied in that it did not infect cucumber or *Cucurbita moschata*. It is designated herein as wild cucumber mosaic virus (WCMV). Five other isolates studied form a natural group and are considered to be strains of another distinct virus, designated as melon mosaic virus (MMV). These isolates included 1 from Wisconsin isolated from muskmelon (RMV); cantaloupe mosaic virus from California (WMV); an isolate collected on squash in Florida (FMV); and 2 isolates (watermelon mosaic virus and yellow watermelon mosaic virus) collected on *Cucurbita pepo* var. *melopepo* in Florida (AMV and YMV).

It should be possible to separate and identify one or the other of the 3 viruses from other viruses that might be present in infected cucurbits in the field on the basis of physical properties, insect transmission, and host range. The first step would be to inoculate Small Sugar pumpkin and tobacco plants. If both hosts became infected systemically, presence of CMV and/or TRSV would be indicated, whereas if only pumpkin were infected, neither of the 2 last-named viruses could be present. If both pumpkin and tobacco were infected, the next step would be insect transmission tests. Beetle transmission to pumpkin would indicate that TRSV, CV3, CV4, and MMV were not present. CMV, SMV, and/or WCMV might still be present, although the recent work of Freitag (8) indicates that CMV is not beetle transmitted. In any case, if the extract from infected pumpkin plants that had been inoculated by means of beetles were aged *in vitro* at room temperature for 28 days, CMV would be

eliminated and inoculation of cucumber and watermelon would separate SMV and WCMV if either were present. If aphid transmission to pumpkin from the original unknown source were successful, TRSV, CV3, CV4, SMV, and WCSV could not be present, but MMV and/or CMV might be. Mechanical inoculation at moderate temperatures to watermelon from aphid-inoculated pumpkin would eliminate CMV; whereas, MMV, if present in the original source, would incite systemic mottle.

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POLYPHENOL OXIDASES AND SUBSTRATES IN POTATO AND TOMATO STEMS[1]

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SUMMARY

Polyphenol oxidase systems were studied because they appear to be involved in the wilt syndrome. Preparations from both potato and tomato stems oxidize phenols that have a second hydroxyl ortho but not meta or para to the first. Preparations from potato stems oxidize tyrosine quickly; those from tomato stems oxidize tyrosine slowly.

The polyphenol oxidase substrate chlorogenic acid is present in easily detectable quantities in tomato leaves and in potato tubers, but it could not be detected in tomato or potato stems. Other substrates present in the stems were differentiated by R_f, fluorescence, and the products formed with diazotized sulfanilic acid and with oxidase. All substrates, however, were present in low concentrations. Therefore, if vascular discoloration results from the action of polyphenol oxidase, substrate not normally found in stems must be liberated during pathogenesis.

Discoloration of the vascular bundles of the stem is a prominent symptom of fungal wilts of potato and tomato. This discoloration has been attributed to the action of polyphenol oxidase (3). Although this enzyme exists at all times in the stem, discoloration is a symptom of disease and does not occur to the same extent in healthy plants. Davis et al (3) have attributed this difference to the freeing of phenols from conjugated phenols during disease development. This would provide substrate in the diseased plant that is lacking in the healthy one. In order to examine this point further, a study has been made of the substrate specificity of the enzymes and substrates present in healthy stems.

Enzyme specificity.—Preparations were made by homogenizing thick peels of Katahdin potato (*Solanum tuberosum* L.) tubers or the stems of 3-week-old Bonny Best tomato (*Lycopersicum esculentum* Mill.) seedlings. Ten g of tissue were homogenized in 30 ml of 0.1 M phosphate buffer (pH 7) at 0° C. Following centrifugation, the clear supernatants were stored in the cold.

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Possible substrates were spotted on No. 3 Whatman filter paper (5). Each spot contained 0.15 micromole of test compound. The papers were sprayed with a solution of 0.5 ml potato preparation or 1.0 ml tomato preparation plus 2 ml of 0.1 M phosphate buffer (pH 6.3). The papers were incubated in a moist chamber and observed for color production.

The results tabulated in Table 1 show the presence in potato tuber peel of the well-known "tyrosinase" (7) capable of oxidizing the monophenol tyrosine and polyphenols with the second hydroxyl ortho to the first. The tomato polyphenol oxidase produced pigment from the same polyphenols as did the potato enzyme but unlike the potato enzyme failed to form a visible amount of pigment from tyrosine. The ability of tomato oxidase to act upon tyrosine was investigated further. One-half ml of a raw or boiled preparation from stems was mixed in 1 millimolar tyrosine, 0.01 millimolar catechol, or a combination of the two to give a total volume of 1.5 ml, pH 6.6. The raw preparation soon formed pigment from tyrosine plus catechol but not from tyrosine alone. A negligible amount of pigment was formed from catechol alone. Thus the only difference between the potato and tomato enzymes is the slow rate at which the latter oxidizes tyrosine unless it is primed by an ortho-polyphenol.

SUBSTRATES IN TOMATO STEMS.—The relative quantities of substrates for polyphenol oxidase in roots, stems, and leaves were examined first. Ten g fresh weight of each organ were obtained from 5-week-old Bonny Best seedlings. The tissue was homogenized in 10 ml of 0.3 M HCl to prevent auto-oxidation. Possible substrates in the acidic solutions were extracted with 2 10-ml lots of ethyl acetate, which was then evaporated to 1 ml by boiling. One-twentieth ml of each extract was spotted on No. 3 Whatman filter paper and developed overnight in the organic phase of a 4:1:5 mixture of butanol, acetic acid, and water. When the chromatogram was sprayed with a solution of potato-peel polyphenol oxidase in 0.1 M phosphate buffer (pH 6) for the identification of substrates, a standard of 0.25 micromole chlorogenic acid was clearly visible.

TABLE 1.—*Relative intensities of pigment formed in 90 minutes by tomato-stem oxidase and by potato-tuber oxidase sprayed upon 0.15 micromole of possible substrates at pH 6.3*

Location (or number) of hydroxyls	Possible substrates	Relative intensities[a] produced by indicated enzyme		
		Potato	Tomato	None
(Mono)	Tyrosine	2	0	0
(Mono)	3-4-Diiodotyrosine	0	0	0
Ortho	3-4-Dihydroxyphenyl-alanine	6	5	1
Ortho	Catechol	4	2	0
Ortho	Chlorogenic acid	3	4	0
Ortho	Pyrogallol	5	3	2
Ortho	Gallic acid	1	1	0
Meta	Resorcinol	0	0	0
Meta	4-Chlororesorcinol	0	0	0
Para	Hydroquinone	0	0	0

[a] Intensity of color produced by each enzyme was ranked from 0 (for none) to the highest number listed (for greatest).

In leaf extract, about 0.1 micromole chlorogenic acid was easily identified by its R_f, fluorescence under ultra violet light (254 mμ), visible yellow color in ammonia vapor, and positive reaction with both potato oxidase and modified Folin-Denis reagent (4). Two additional fluorescent substrates were shown at R_f 0.52–0.56 and 0.44–0.47 by their reaction with potato oxidase. This positive result shows that the extraction does not destroy all of the substrates.

In contrast to the abundance of chlorogenic acid and other substrates present in tomato leaf extract, no substrates were found in either the stem or root extract.

Having established the relative scarcity of polyphenol oxidase substrates in tomato stems, we next attempted to determine whether any substrate is present at low concentrations in several varieties of tomatoes grown under both outdoor and greenhouse conditions. Two hundred twenty g wet weight of Bonny Best, Pritchard, and Pan American stems and roots were collected from 5- and 13-week-old plants in the greenhouse and from 5-month-old plants outdoors. All of the tissue was ground together in 600 ml ethanol and the extract evaporated to 5 ml *in vacuo* at less than 40° C. This brown solution was washed with 2 10-ml lots of petroleum ether. When this water solution was chromatographed, no substrates could be definitely identified. Therefore, it was concentrated by extracting with an equal volume of ethyl acetate, which was then evaporated to 0.5 ml. This solution was washed with an equal volume of water. One-twentieth-ml lots of this concentrate were placed on filter paper and developed in the mixture of butanol, acetic acid, and water as described.

Chlorogenic acid could not be identified positively, even in this concentrate. If equal yields from leaf and stem extraction are assumed, this failure to find chlorogenic acid in the stem means that its concentration in the stem is less than 1/40 its concentration in the leaves. Other substrates were seen, however. One substrate (T_1), with an R_f of 0.91, turned tan when sprayed with potato polyphenol oxidase and formed a darker tan addition product when sprayed with both oxidase and phloroglucinol. T_1 formed a red-brown compound when sprayed with diazotized sulfanilic acid (1, p. 95). T_1 was not fluorescent, nor did it become visible in ammonia vapor. A second substrate (T_2), with an R_f of 0.88, was fluorescent, became tan when oxidized in the presence of phloroglucinol, and formed a red-brown pigment when sprayed with diazotized sulfanilic acid. Both substrates were present in very low concentrations, producing much less pigment than 0.25 micromole of chlorogenic acid.

SUBSTRATES IN POTATO STEMS.—An extract from 200 g fresh weight of field-grown Kennebec potato stems was prepared by the same method as for the large

tomato stems except that the final wash with water was omitted. One-twentieth-ml lots of the extract were placed on paper and developed in butanol: acetic acid: water.

Chlorogenic acid is known to be present in potato tubers (4). As a check on our methods, we tested tubers and found chlorogenic acid. Nevertheless, this substrate was not detected in the extract of green above-ground potato stems. If equal yields from stem and tuber extracts are assumed, this failure to find chlorogenic acid means that its concentration in the stem is less than 1/40 its concentration in tuber peel.

Two substrates were detected in potato stems. P_1, at an R_f of 0.87–0.90, turned tan when sprayed with oxidase and intense orange when oxidized in the presence of phloroglucinol. P_1 formed an orange-brown compound when sprayed with diazotized sulfanilic acid. It was fluorescent, and it did not become visible in ammonia. The orange compound that formed when P_1 was oxidized in the presence of phloroglucinol differentiates it from T_1 and T_2. P_2, with an R_f of 0.76-0.80, also formed an orange-brown product when sprayed with diazotized sulfanilic acid, was fluorescent, and did not turn yellow in ammonia. Unlike those of T_1, T_2, and P_1, the oxidase product was darker in color when phloroglucinol was absent than when it was present.

An attempt was made to obtain an extract of higher polyphenol concentration by extracting a second sample composed of only the outer portion of Kennebec stems. An ethanolic extract was again prepared and evaporated. It was then acidified and extracted with ethyl acetate, which was then washed with acidified water. The ethyl acetate extract from 210 g fresh weight was then evaporated to 3 ml. P_1 and P_2 were again identified on a chromatogram developed in butanol: acetic acid: water, but chlorogenic acid was not. Both substrates were present in very low concentrations, producing much less pigment than 0.25 micromole chlorogenic acid.

Discussion.—Peroxidases, capable of oxidizing polyphenols, could confuse the determination of substrates by the spot technique. Had this occurred in the test summarized in Table 1, polyphenols with the second hydroxyl meta or para to the first would have been oxidized. This did not occur, perhaps because of a lack of peroxide.

The slowness with which polyphenol oxidase from healthy tomato stems oxidizes tyrosine (Table 1) was observed earlier (6). This slowness distinguishes the tomato enzyme from the potato enzyme. Variations among such enzymes in relative activity on monophenols and polyphenols is common (7); the tomato stem enzyme has a low relative activity on monophenol and requires priming (8) to be easily detectable.

Tyrosine is oxidized by tyrosinase by first being converted to 3,4-dihydroxyphenylalanine (dopa), according to the generally accepted hypothesis. The failure of the enzymes to oxidize 3,5-diiodotyrosine is in agreement with this hypothesis. We assume that a hydroxyl cannot be substituted at the 3 position already occupied by I. Hence, the formation and subsequent oxidation of dopa is prevented as suggested (2) for other monophenols with substituents in the ortho position.

In the spot test (Table 1), the tomato-stem enzyme produced only a faint amount of pigment from gallic acid and none from resorcinol. In contrast to this, Davis et al (3) obtained a dark pigment from gallic acid and some pigment from resorcinol taken into the vessels of tomato cuttings. This difference in results suggests that these 2 phenols produce vascular discoloration indirectly rather than through their enzymatic oxidation.

Although the examination of tomato stems and roots and of above-ground potato stems for polyphenol oxidase substrates has not been carried to the point of identification of the substrates present, observations of significance in the physiology of tomato and potato wilt were made. Chlorogenic acid, the polyphenol oxidase substrate present in easily detectable quantities in tomato leaves and potato tubers, was not found in the stem regions that show vascular discoloration. Also, the substrates found were present in small amounts. Therefore, if the hypothesis that vascular discoloration is attributable to polyphenoloxidase (3) is correct, the discoloration must be due to the liberation during pathogenesis of amounts, and possibly kinds, of polyphenolic substrates not normally found in stems. The suggestion (3) that substrates are liberated by hydrolysis of conjugated phenols is a probable one because hydrolytic enzymes are known to be produced in the host by the pathogen.

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SEROLOGICAL STUDIES OF VIRUSES INFECTING THE CEREAL CROPS. I. A COMPARISON OF BARLEY STRIPE MOSAIC VIRUS AND BROME MOSAIC VIRUS ISOLATES BY MEANS OF THE COMPLEMENT-FIXATION TECHNIQUE [1]

Ellen L. Moorhead [2]

SUMMARY

Serological studies of brome mosaic virus (BMV) and barley stripe mosaic virus (BSMV) are reported. Antigen preparations were purified and concentrated by high-speed centrifugation. Rabbits were injected intravenously with saline suspensions of the virus and intramuscularly with an emulsion of virus and mineral oil (adjuvant). Periodic bleedings were made to trace the course of antibody production. Cross-reaction studies on serum samples collected during the primary phase of antibody production and those collected after a booster dose showed no alteration in the specificity of the immune reaction. No cross reactions were observed between BMV and BSMV, which indicates that these viruses are distinct serological entities. Of the 7 isolates of BSMV and 4 of BMV, no strain differences could be detected by the complement-fixation technique, indicating that these isolates are the same or are closely related to the "type" strain.

Viruses causing mosaic symptoms have been isolated from wheat (3, 5), rye (1), oats (4), brome grass (8), and more recently from barley (6). At least 2 strains of wheat soil-borne virus have been described, a green mosaic (*Marmor tritici* var. *typicum* McK.) and a yellow mosaic (*M. tritici* var. *fulvum* McK.) (2). An eyespot mosaic virus (*Marmor terrestre* var. *oculatum* McK.) and an apical mosaic virus (*M. terrestre* var. *typicum* McK.) have been isolated from oats; both strains are soil borne (4). Variation in symptom expression of natural field-isolates suggests the possibility that barley stripe mosaic virus also may comprise several strains. Though many similarities and differences exist among these cereal viruses with respect to host range and chemical and physical properties, their exact interrelationships are not clear. Nor is it certain that a particular virus isolated in the southeastern United States is the same as, or closely related to, one found in the Great Plains region. In order to provide information on the relationships among the cereal viruses, investigations on their fundamental characteristics have been undertaken. Since a number of plant viruses have been shown to be antigenic, it seemed probable that the serological approach might give an insight into the nature of the cereal viruses and also serve as a means of identifying them in infected plant tissue. This series of papers describes the results obtained from a serological study of a number of cereal viruses.

It has been shown recently (7) that brome mosaic virus occurs naturally on field-grown barley and that barley stripe mosaic virus may be largely responsible for some of the low barley yields. Differentiation of these 2 viruses can be made on the basis of symptom expression in such host plants as *Zea mays* L. var. Golden Giant Sugar, *Phaseolus vulgaris* L. var. Scotia, *Chenopodium album* L., *Cucumis sativus* L. var. Early White Spine, and on the basis of such physical properties as thermal inactivation point and infectivity dilution endpoint (7). These 2 viruses were selected for the initial serological study because of their economic importance and the possibility that they may be widely distributed in nature. The results obtained from producing antiserum specific for these viruses are presented here.

MATERIALS AND METHODS.—*Virus isolates under investigation.*—Seven isolates of barley stripe mosaic virus (BSMV) and 4 of brome mosaic virus (BMV) were studied. Antisera specific for the type strain of BSMV (ATCC #69) and to a California isolate obtained from A. H. Gold, University of California at Berkeley, were prepared in rabbits. These sera were used for comparison with their own antigens and with 5 other isolates obtained from H. H. McKinney, Beltsville, Maryland, and from R. G. Timian, Fargo, North Dakota. Also, antisera were prepared to 2 isolates of brome mosaic virus (BMV), *Marmor graminis* McK; one was to the type strain (ATCC #66), and the other to a greenhouse isolate referred to as BMV1. Cross-reaction studies were made between these sera and 2 isolates obtained by H. H. McKinney from naturally infected plants in the field.

Preparation of antigens.—Infectivity dilution endpoints of BSMV and BMV confirmed McKinney's observations that BSMV was present in host plant tissue in relatively low concentrations as compared to BMV (7). The viruses were increased in barley plants (*Hordeum vulgare* L. var. Moore). Seedlings in the 2-leaf stage were inoculated by the leaf-rubbing method (5). Approximately 2 weeks after symptoms appeared, the plants were harvested and ground in an

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ordinary meat chopper. The pulp was stored in wax-lined ice-cream cartons at −20°C. Infectious plant sap was expressed from the thawed pulp through several layers of cheesecloth and then concentrated and purified by alternate low- and high-speed centrifugations. Two centrifugations at 4,000 G for 30 minutes separated by 1 centrifugation at 78,000 G for 3 hours comprised 1 complete cycle. One or 2 cycles were used to prepare the barley stripe mosaic viruses and 3 cycles for the brome mosaic viruses. The BSMV pellets from high speed centrifugation were resuspended in 0.05 M phosphate buffered saline, and those of BMV were resuspended in distilled water. Electrophoretic mobilities and serological cross reactions with serum prepared from plant constituents established the purity of the antigens.

The weight of virus in each antigen preparation was determined by bringing a known volume to constant weight in a hot air oven at 105–110°C. The final virus preparations were passed through a Seitz filter and stored at −20° or 4°C in rubber-capped serum vials. Storage under these conditions failed to change the antigenic units as established by serological tests.

Preparation of antisera.—In every case in which purified virus was used, an attempt was made to inject the same weight of virus into each rabbit. Four intravenous injections of the virus suspension were given in small volumes, 0.1–2.0 ml each week, until a total of approximately 20 mg had been injected. Every 2 weeks, bleedings were made by the cardiac-puncture technique to trace the antibody response. When a marked decrease in antiserum titer was ascertained by serological tests, a booster dose of 6–10 mg of antigen emulsified in mineral oil (adjuvant) (11) was injected into the thigh muscle of the rabbit. On 2 occasions, 1 of an immunized pair of rabbits died during the antibody-production period; other rabbits were substituted, but each was given only a single injection of 20 mg of virus in an adjuvant.

A sample of blood for normal control serum was removed from each rabbit before any injections were given. Small samples of blood, less than 10 ml, were withdrawn at each bleeding. The blood was allowed to clot, rimmed to free the clot from the tube, incubated at 37°C for 1 hour, and held at 4° overnight. The serum was removed and freed of cellular material by centrifugation. All sera were stored in rubber-stoppered vials at −20° until used in serological tests.

Serological test procedure.—The sera were tested by the complement-fixation technique (12),[3] which is an accurate and sensitive method for detecting similarities or differences among strains of several of the plant viruses (10, 12, 13). In these tests, only reaction endpoints indicative of complete fixation were considered significant.

[3] Sheep red blood cells from Brown Laboratories, Topeka, Kansas. Complement and antisheep hemolysin from Sharp & Dohme, Philadelphia, Pennsylvania.

Grid titrations of each homologous antigen antibody system were prepared by mixing 2-fold serial dilutions of antigen with a similar series of antiserum dilutions. The optimum reacting dilution of each antigen was determined as previously described (10, 13). This dilution of antigen was used to define the antibody limits of each serum collected throughout the production period in each rabbit and also to test for cross reactions in the sera prepared to other viruses.

EXPERIMENTAL RESULTS.—The maximum antibody response in rabbits immunized with the BSMV antigens (ATCC #69 and California isolate) occurred 2 weeks after the initial injection of virus in 0.05 M phosphate buffered physiological saline, pH 7.0 (Fig. 1, A). A steady decline in antibody production followed, and a booster dose of virus in an adjuvant given in the sixteenth week produced a rapid increase in antibodies. Of this group, 2 rabbits that were immunized with a single injection of virus in an adjuvant did not produce a maximum response until the sixth to eighth week, but the antibody response remained

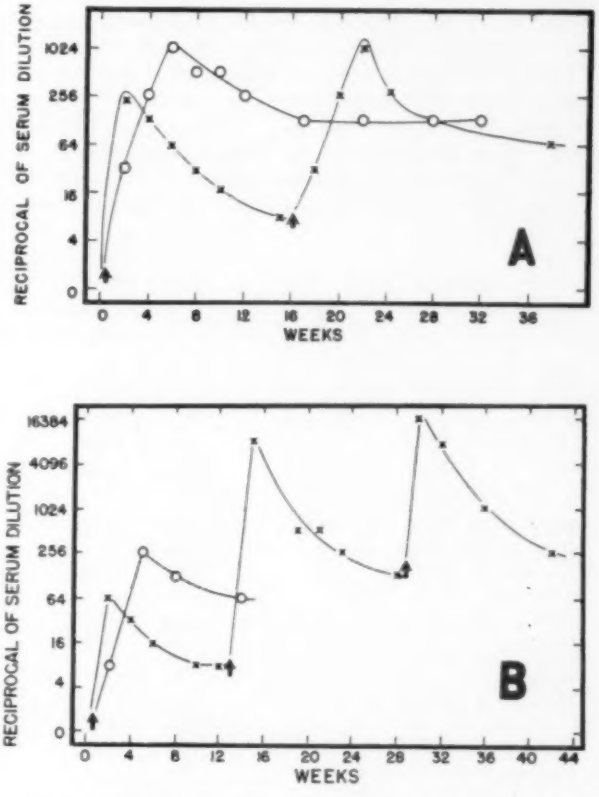

FIG. 1. Antibody response in rabbits to injections of 2 cereal viruses. Open circles: Primary injection of virus was in an adjuvant. Crosses: Primary injection of virus was in 0.05 M potassium phosphate in 0.85 per cent NaCl, and the booster dose was given in an adjuvant. The arrows indicate when injections were given. A) Immunization with barley stripe mosaic virus. B) Immunization with brome mosaic virus.

high for a longer time than with the saline injections.

Likewise, rabbits injected intravenously with BMV or BMV1 antigens suspended in distilled water, pH 6.0, showed a maximum response in 2 weeks (Fig. 1, B). An adjuvant-virus booster dose in the thirteenth week gave a high-titer antiserum, but the maximum response period was short. A rabbit receiving a single dose of BMV1 in an adjuvant gave a maximum antiserum titer in 5 weeks.

Cross-reaction studies were made on antiserum obtained during the primary response period and on antiserum obtained after an effective booster dose was given. Hyperimmunization did not alter the specificity of the reaction, since the relationships established by early serum samples were present to the same extent after the booster dose.

A summary of the serological relationships among isolates of these 2 cereal viruses is given in Table 1. In tests with the complement-fixation technique, differences greater than a 2-fold dilution must be present to be significant. The individual rabbits showed some variation in their response to a given virus, but this was considered normal. No cross reaction between the 2 major viruses was observed, indicating that BMV and BSMV are distinct serological entities. Also, differences among the isolates of each major virus could not be detected by the complement-fixation technique. The isolates from naturally infected plants reacted with the type strain antiserum to the same extent as did the homologous type antigen. This would indicate that these field isolates are either identical with or very closely related to the type strain.

Discussion.—The experimental results obtained from this study of 2 cereal viruses indicate that the viruses can be freed of contaminating plant material and concentrated by high-speed centrifugation and that they are stable with respect to their serological titers in the purified form when stored at cold temperatures. These purified preparations react as satisfactory antigens when injected into rabbits, whether the inoculum is suspended in physiological saline or in a mineral oil emulsion. It seems preferable to adopt the adjuvant method of immunization for future sera preparations of these viruses, since these injections are less troublesome than repeated saline injections, since at least 4-fold higher maximum antiserum titers can be obtained from the initial injection, and since a higher over-all level of antibody can be maintained for a longer period of time.

Because a booster dose of virus in saline was not given, it can not be said at this time how effective such an injection might be; however, the booster inoculation of BSMV in adjuvant increased the maximum titer only 4-fold, whereas the maximum titer with BMV was increased 1000 times over that from the primary saline injections. It is interesting that the maximum peak of antibody production was maintained for only a short period of time even when adjuvant was used. This is in direct contrast to immunity studies with tobacco mosaic virus and with southern bean mosaic virus in adjuvant where a high level of response, a dilution endpoint of 1:512 or 1:1024, was maintained for 30 or more weeks (9). Also, with these cereal viruses the phase of rather constant antibody production appeared at the same titer, a dilution of 1:128, whether the adjuvant injection was a primary or a

Table 1.—*Reciprocal of the dilution endpoints of antisera specific for brome mosaic virus (BMV) and for barley stripe mosaic (BSMV) when tested by the complement-fixation technique against purified preparations of various isolates of the 2 viruses*

Antigens and source[a]	Dilution endpoints[b] of indicated antisera								
	BMV ATCC #66		BMV1		BSMV ATCC #69		BSMV California		Barley plant sap
	Rabbit No. 7	Rabbit No. 8	Rabbit No. 3	Rabbit No. 4	Rabbit No. 11	Rabbit No. 12	Rabbit No. 1	Rabbit No. 2	(Rabbit No. 5)
BMV Type ATCC #66, H. H. McK.	1024	512	256	128	<4	<4	<4	<4	<4
BMV1, H. H. McK.	1024		256		<4		<4		<4
Lincoln, H. H. McK.	1024	1024	256	128	<4		<4		
Manhattan, H. H. McK.	1024	1024	256	128	<4		<4		
BSMV Type ATCC #69, H. H. McK.	<4	<4	<4	<4	128	1024	256	1024	<4
California, A. H. G.	<4	<4	<4	<4	128	1024	256	1024	<4
Glacier #2 (Mild), H. H. McK.	<4		<4		128		512		
Hagborg, R. G. T.	<4		<4		256		256		
Manchuria CI 2330, R. G. T.	<4		<4		128		256		
California "E," R. G. T.	<4		<4		128		256		
McKinney's 52-1, R. G. T.	<4		<4		128		256		
Healthy barley plant sap	<4	<4	<4	<4	<4	<4	<4	<4	256

[a] Virus antigens were purified and concentrated by high-speed centrifugation and were suspended in distilled water (for BMV isolates) or 0.05M potassium phosphate buffer in 0.85 per cent NaCl (for BSMV isolates). The optimum reacting dilution of each antigen was determined by grid titrations of homologous antigen and antiserum, and this concentration of antigen was used to determine the extent of cross reaction with heterologous sera. Virus sources are indicated by the initials of the investigator: H. H. McK.—H. H. McKinney, Beltsville, Maryland; A. H. G.—A. H. Gold, Berkeley, California; R. G. T.—R. G. Timian, Fargo, North Dakota.

[b] Since anticomplementary results occur in dilutions less than 1:4, sera giving these low endpoint readings were considered negative in cross-reaction studies.

booster dose and regardless of the extent of the maximum antibody titer. Virus concentration and stability, as well as the continued state of the emulsion of mineral oil and virus are important contributors to the shape of the antibody curve, but the nature of the viruses, the methods used for immunization, and the individuality of the rabbits also attribute to the characteristics described here. Further studies of the immune response of rabbits to cereal viruses may clarify some of these results.

Of importance in the cross-reaction studies is the fact that BMV and BSMV are separate and distinct viruses. Since no cross reactions with normal plant proteins or with the other viruses were observed, these sera may be used in the field to identify the homologous virus and to eliminate the cumbersome laboratory and greenhouse tests that otherwise must be used for such identification. A simple serological test has been devised for these viruses and will be reported in another paper.

The grid titrations of each homologous antigen-antibody system established the optimum reacting dilution of antigen and permitted a calculation of the weight of virus reacting in these dilutions. This figure is specific for each antigen and is referred to as the optimum antigenic unit. Since it has been shown that strains of tobacco mosaic virus can be differentiated on the basis of the optimum antigenic unit (9, 10, 13), a similar attempt was made to correlate the results of the present study. The optimum antigenic unit for the BMV isolates was of the order of 2.0 to 8.0 mg and that for the BSMV type strain and the California isolate were approximately 22.0 mg. Since the other BSMV isolates were not purified to the same extent as the 2 used for antibody production, their optimum antigenic units were not determined at this time. Further experimental studies are necessary to establish relationships among strains of these cereal viruses on the basis of the optimum antigenic unit.

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ELIMINATION OF FOLIAGE SPOTTING FROM SWEETPOTATO [1]

Francis O. Holmes

SUMMARY

A stock of sweetpotato plants, all of which showed chlorotic spotting of foliage like that generally associated with internal-cork disease, was transformed into a stock free from the leaf abnormality. This was effected by grafting short stem tips of the diseased plants into the hypocotyls of seedlings of Heavenly Blue morning glory and of Giant White moonflower. When the originally small scions had grown to a length of several inches, those that showed unspotted green foliage were selected and established independently as rooted cuttings. All others were discarded. Although the selected plants were susceptible to reinfection if grafted with diseased scions, they remained free of the leaf-spotting disease when grown in the absence of a source of reinfection.

Chlorotic blotches and ringlike patterns on leaves of sweetpotato, *Ipomoea batatas* (L.) Lam., were observed in all young plants grown from a stock of roots of the Bunch Porto Rico variety. Such foliage abnormalities appeared to be common in this variety, although they were not constantly in evidence even in stocks known to show them at times. The leaf markings resembled lesions that have been described as associated with internal-cork disease.[2,3,4] Patterns typical of those seen in this stock of sweetpotato are depicted in Figure 1, A. Their scattered distribution on the leaves of the diseased plants suggested that the causative virus might tend to be localized rather than uniformly distributed in the tissues of sweetpotato and that some of the very young parts near the tips of the shoots might be free from the disease.

An earlier success in restoring to health a stock of dahlia, *Dahlia pinnata* Cav., all available plants of which were chronically affected in a somewhat similar way by spotted-wilt disease,[5] suggested that the diseased stock of sweetpotato might be transformed into a healthy clone by propagation of tip cuttings from affected branches. Rooting of small stem-tip cuttings had been adequate in the case of the dahlia.[6] In the sweetpotato, however, chlorotic spots were noted in leaves so close to growing points that stem-tip cuttings large enough to be rooted, even if only an inch or so in length, would have included the affected parts.

In order to use cuttings smaller than could be rooted readily, stem tips were used as scions. Usually the tip cuttings were about ¼ in. long and included only 1 leaf longer than ⅛ in. These were grafted onto healthy young seedlings of the morning glory, *Ipomoea tricolor* Cav. (var. Heavenly Blue), or onto healthy seedlings of moonflower, *Calonyction aculeatum* House (var. Giant White). These seedlings were regarded as more likely to constitute virus-free understocks than any vegetatively propagated plant would be. They were used on this account in preference to supposedly healthy lines of sweetpotato.

The understocks were prepared originally by removal of a cotyledon and the main shoot from each seedling. By this procedure, the subsequent growth of side branches of the understock was delayed. The remaining cotyledon provided sufficient leaf surface to feed the stem and root system adequately. Each scion was inserted in a cleft cut at the top of the hypocotyl, as shown in Figure 1, D. Adventitious buds developed eventually on the understock at the cotyledonary axil near the inserted scion. Shoots developing from these buds were difficult to remove without injury to the sweetpotato tissues. Subsequently, scions were inserted lower on the stem to facilitate removal of any developing axillary shoots of the understock. When this was done, both cotyledons could be left on each seedling to feed the stem and roots. A downward slanting cleft was made in each hypocotyl about half way between the cotyledonary level and the root. Then a very small sweetpotato stem tip was cut to a wedge shape, inserted in the cleft, and held in place there by 2 opposing squares of tacky crepe rubber. The squares of crepe rubber were stretched slightly and pressed firmly together. The completed sweetpotato-on-morning-glory graft is illustrated in Figure 1, E.

Sweetpotato scions sometimes failed to become established, but generally they grew well. A few successfully grafted plants showed chlorotic spots in developing foliage of the sweetpotato scions; these were discarded on the assumption that they represented failures to exclude the original virus.

Scions that grew well and showed no chlorotic spots on their leaves were used subsequently as a source of stem cuttings large enough to be rooted readily. These cuttings, after they had produced roots of their own, were established as separate young sweetpotato plants during the spring of 1953. None of them showed chlorotic spotting of foliage during the ensuing summer and autumn, but this was not regarded as

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FIG. 1. Bunch Porto Rico sweetpotato. A) Leaf from a diseased plant, showing chlorotic spots, especially along veins. B) Leaf from the same stock as A after its restoration to health. C) Leaf from a cutting plant after its reinfection by grafting. D, E) Successful grafts of small sweetpotato scions on Heavenly Blue morning glory understocks, in D at the top of the hypocotyl, in E half way down. The crepe rubber squares that held the scions in place until union occurred have been slipped down to show the V-shaped junctions between scions and understocks. The original morning glory cotyledons can be recognized, at the right in D and high at the left and right in E; all other leaves were developed on the scions. Photographs by J. A. Carlile.

significant because the diseased stock from which they had been derived showed only normal or nearly normal-appearing green leaves during this part of the year.

Eight of the lines that had been propagated first as grafted scions, and later as cuttings on their own roots, were maintained as single or duplicate plants in 6-in. pots through the winter of 1953–1954. One of a pair of duplicate plants showed chlorotic spots on 3 leaves early in February. These leaves were removed at once, but the plant was kept for further observation. No other leaves on this or the remaining plants showed chlorotic lesions for about 6½ weeks. Then 2 plants immediately adjacent to the symptomless individual that had shown chlorotic spots in early February displayed widespread spotting of foliage. All 3 diseased plants were removed immediately to another greenhouse to avoid spread of the disease to the remaining healthy plants. No further spread of the disease was noted through the following spring and summer months.

Later, the plants that had shown no disease subsequent to their propagation from diseased plants were used as sources of cuttings for further propagation. During the next winter a much expanded series of sublines was maintained in triplicate. One plant of each sub-line was grafted with a long-stemmed scion from a diseased but symptomless plant. The remaining 2 of each sub-line of 3 cutting-plants were grown without further treatment, as control plants. The scions used in grafting were chosen with a view to including tissues old enough to carry the disease. Altogether 26 plants were thus grafted, some of them repeatedly. During the spring and summer months, 13 of these showed characteristic chlorotic spots on their leaves, indicating susceptibility to reinfection. A few others showed a trace of chlorotic spotting but not enough to identify the disease with certainty. None of the 52 control plants that were not grafted showed similar chlorotic spots, but 1 died before the observations were concluded. The 13 grafted plants that failed to show characteristic chlorotic leaf-spotting may have become infected without showing chlorotic spots at the time of onset of disease, possibly passing at once, or with symptoms so faint as to be unrecognizable, into the chronic state of disease in which leaf spotting often is absent or too slight for positive identification.

Subsequently a similar experiment was performed with 12 subclones from the same healthy stock that had been freed of the leaf spotting. One plant of each subclone was grafted with a long scion cut from a chronically diseased but symptomless plant. Two additional plants of each subclone were maintained as control plants. About 2½ weeks after inoculation by grafting, 5 of the 12 grafted plants showed characteristic chlorotic spots on recently developed leaves. Two additional plants showed questionable lesions a few days later but the 5 remaining plants failed to show any sign of the disease for some time. Much later (9 weeks after inoculation by grafting) a sixth plant showed clear-cut indications of infection for the first time. The 24 control plants appeared healthy throughout the course of the experiment.

A leaf from one of the healthy lines produced by grafting tips of sweetpotato stems onto morning glory seedlings is shown in Figure 1, B, for comparison with the diseased leaf represented in Figure 1, A. A leaf from a similar plant after its reinfection by grafting is shown in Figure 1, C.

Evidence that a leaf-spotting disease had been eliminated by excision and propagation of small tips of branches was provided by the production of the original type of disease in 2 experiments involving reinfection by grafting and the continued absence of such a disease in isolated control plants. What relationship may exist between this foliage-spotting disease and the root disease known as internal cork of sweetpotato is still in question. Frequent association of the foliage and root diseases suggests that they may be separate results of infection by a single virus. Success in eliminating the foliage-spotting disease by propagation of small scions from stem tips provides a method for determining whether these diseases have a common cause. Investigators having facilities for study of both diseases may be able eventually to compare the incidence of the root disease, internal cork, in clones of sweetpotato plants before and after elimination of the foliage-spotting malady.

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HOST-PARASITE RELATIONSHIPS OF THE LANCE NEMATODE IN COTTON ROOTS [1]

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SUMMARY

Cotton plants growing in the field under low moisture conditions were severely stunted, yellowed, and almost completely defoliated in areas heavily infested with the lance nematode, *Hoplolaimus coronatus*. Populations of the nematode increased on cotton in the greenhouse with little stunting of the plants. Seed germination was not affected by the nematode. In the absence of a host plant, the nematode did not reproduce. It apparently cannot live as a saprophyte.

H. coronatus was observed feeding both ectoparasitically and endoparasitically on cotton roots. When feeding endoparasitically, the nematodes lay in the cortex longitudinal to the long axis of the root, 2–3 cell layers beneath the epidermis. It fed in both the cortex and vascular tissues. Phloem seemed to be the most preferred root tissue; little if any feeding occurred on xylem. Injury was much more extensive in the vascular cylinder than in the cortex. The staining characteristics of injured vascular cells indicated an alkaline reaction. Many tyloses were produced in injured xylem elements.

The lance nematode, *Hoplolaimus coronatus* Cobb, 1923, although not generally regarded as a serious plant parasite, often has been reported in association with or feeding upon various plants. Some of these include rice (1), bulbs (3), *Pinus* sp. (4, 12, 14, 15), citrus (5, 17), peach (6, 12), lawn grass (7, 20), cotton (9), soybean (9), sugarcane (9, 10, 13), corn (13), red clover (13), alfalfa (13), boxwood (15), elm (18), pin and red oaks (18), red cedar (18), holly (18), and Chinese water chestnut (19).

In August of 1954, in Johnston County, North Carolina, roots of severely stunted, yellowed, and almost completely defoliated cotton plants were found to be heavily infested with lance nematodes. Preliminary screening of soil collected from the rhizospheres of both diseased and healthy plants indicated a probable relationship between the lance nematode and the stunted condition of the cotton. This paper reports the results of experiments on host-parasite relationships of the lance nematode. The objectives were to study 1) population levels of this nematode in soil samples collected from near both diseased and healthy plants in the field, 2) pathogenicity and population increase of the lance nematode on cotton under greenhouse conditions, 3) the effect of the nematode on cotton seed germination, and 4) the histo-pathological effects of the nematode on root tissues. A preliminary report on pathogenicity has been given (8).

MATERIALS AND METHODS.—Population levels of the lance nematode in the field were investigated. Pint samples of soil were collected from the rhizosphere of each of 10 plants selected at random in areas where the cotton *Gossypium hirsutum* L. was severely stunted (Fig. 1, A) and of 10 plants in areas where the cotton appeared healthy (Fig. 1, B). The nematodes were removed from the soil for counting by the technique described by Christie and Perry (2). This technique was employed in all subsequent experiments involving the removal of nematodes from the soil.

Test-tube cultures were used to determine the effects of the lance nematode on seed germination and subsequent seedling growth. Ten test tubes (2.5 × 20 cm) were filled to within 1 in. of the rim with moist, autoclaved, fertilized sand. One cotton seed was planted in each tube, and 100 lance nematodes then were placed in close proximity to each seed. Ten test tubes with cotton seeds, but without nematodes, served as controls. In 4 additional tests 200 nematodes per tube and 20 replications were used. The tubes were placed under fluorescent light at 75°F and the plants allowed to grow for 2 weeks. Observations of the nematode feeding on the roots were made through the test-tube walls by means of a dissecting microscope.

The rate of reproduction of the lance nematode on cotton and the effects of the nematode on plant growth were determined in greenhouse tests. Cotton was planted in 18 8-in. pots of soil that had been fumigated with methyl bromide. Nine pots were infested at time of planting, each with 500 lance nematodes, and 9 pots without nematodes served as controls. Greenhouse temperatures ranged from 75–85°F. When the first planting matured, the plants were cut off at the soil level. Cotton was again planted in these pots, and this procedure was repeated 3 times for a total growth period of 344 days. The nematode population was determined for 2 of the infested pots and 2 of the controls after 112 days and for the remaining 7 pots of each series at the end of 344 days.

Histological studies were made of lance nematodes feeding within cotton roots. Material for study was obtained by growing seedlings in infested sand at 75°F under fluorescent light. After 2 weeks' growth, the

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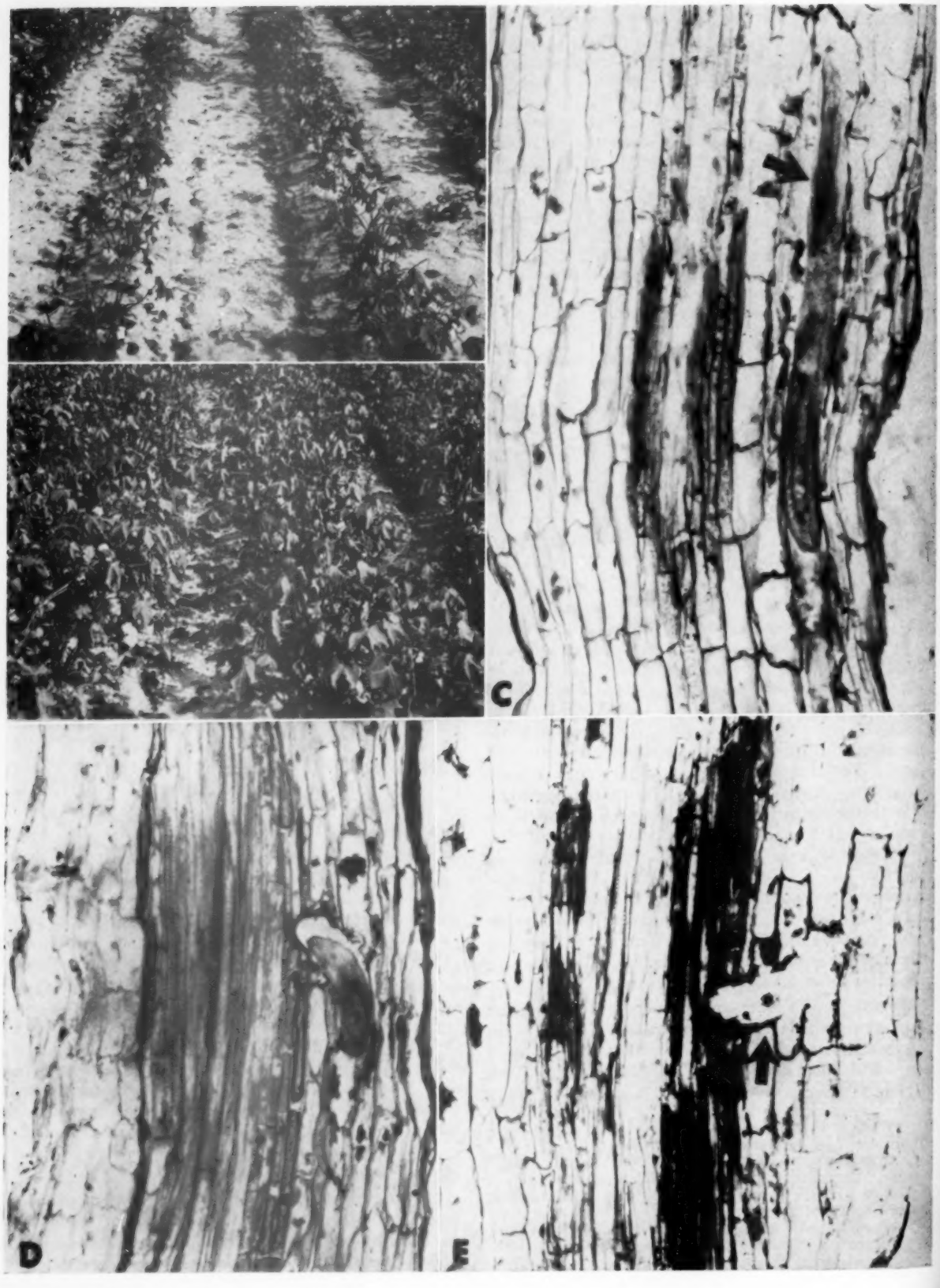
C
D
E

roots were carefully washed and the entire root system fixed in CRAF III for 24 hours (11). The fixed roots were examined microscopically, and those that were nematode-infected were removed for sectioning. An ethanol-xylene series was used for dehydration. Roots were embedded in tissue mat (melting point, 55°–57°C). Both cross and longitudinal sections were cut 10 μ thick. Fifty-five pieces of root were sectioned. Sections were stained in safranin and fast green.

EXPERIMENTAL RESULTS.—*Population levels in the field.*—The soil samples collected from the rhizosphere of stunted and of healthy plants in the field averaged 1495 and 125 lance nematodes per pint, respectively. This difference was highly significant (LSD at 0.01 level, 454). Both larval and adult stages of the nematode were observed. In addition to the lance nematode, a few nematodes of the genera *Helicotylenchus*, *Pratylenchus*, and *Meloidogyne* were recovered.

Pathogenicity and population increase in greenhouse tests.—Germination of cotton seed was not affected by the presence of the lance nematode. The nematodes were observed feeding on seedling roots that were growing along the sides of the test tube. Migration from root to root occurred freely and the nematodes were observed feeding both as ectoparasites and as endoparasites. Definite root lesions were associated with the feeding of the nematodes. A brownish-yellow discoloration occurred in the epidermal cells at the point of nematode penetration and in cells surrounding the path of the nematode through the tissue. The discolored area extended into 2–5 cells around the path made by the nematode.

The first planting of cotton in the nematode-infested soil in the greenhouse showed no stunting, although later plantings showed slight stunting. The population level of the lance nematode after 112 days was 741 (average of 2 pots) per pot and after 344 days, 12,883 (average of 7 pots) per pot.

Histological studies.—1) *Position of the nematode in relation to root tissues.*—When embedded in a root, lance nematodes were situated in the cortex, 2–3 cell layers inside the epidermis and parallel to the longitudinal axis of the root (Fig. 1, C). The nematodes penetrated the root cortex and eventually came to a position with only the anterior portion of the body and lip region curved into the conducting tissue, the rest of the body remaining in the cortex (Fig. 1, D). Sometimes the nematode penetrated the root in a straight line perpendicular to the stele, reaching the vascular system without the entire body becoming embedded in the cortex.

2) *Cortical damage.*—The lance nematode caused considerable cell damage in penetrating the cortex. The passageway formed by the nematode was just large enough for the nematode to pass through, except where it stopped to feed or to lay eggs. Where the nematode had stopped to feed on cortical cells, several cells were collapsed, forming a cavity. Many surrounding noncollapsed cells were devoid of cell contents. Cortical damage was mostly restricted to the area immediately around the nematode, extending 1–4 cells from the nematode.

Cell walls of damaged and deformed cortical cells appeared thicker (Fig. 1, E) and stained deeper with fast green than did undamaged cells. Walls of a few of the damaged cortical cells stained reddish with safranin, like those in vascular tissue. Certain penetrated cortical cells acquired a membrane across the break (Fig. 1, E).

Several nematode eggs were found embedded in the cortex of various roots. All of these were beside the nematode passageway and 2–3 cell layers inside the epidermis.

3) *Endodermal damage.*—The lance nematode penetrated endodermis much like it did cortex. The nematode made a hole in the endodermis large enough for it to feed easily on the vascular tissue (Fig. 1, D, E; 2, A, C) but the cavity was not extensive. Surrounding endodermal cells were not crushed when this feeding cavity was made by the nematode. Damage extended 2–4 cells in all directions from the nematode puncture (Fig. 2, A). Many of the killed cells were outside the area of direct feeding.

A thick membrane similar to that found in certain cortical cells was found in penetrated endodermal cells adjacent to the nematode (Fig. 1, D, E; 2, A).

In cells recently fed upon, the cell contents were granular in texture and gray-green or red in color, with the cell walls stained bright red. Where cells had been damaged a longer period of time, cell walls were a deeper red and the cell contents had disappeared (Fig. 2, A). The stained walls of emptied cells appeared very thick.

4) *Phloem damage.*—In these tests, *H. coronatus* always penetrated to a phloem group and fed on the phloem parenchyma and phloem elements, causing death of these cells. In 1 instance, a nematode penetrated a root from the outside and proceeded directly to a phloem group. Numerous punctures occurred in phloem parenchyma and phloem elements within reach of the stylet of the nematode. In cells recently fed upon, the contents were disorganized and stained

FIG. 1. A, B) Comparison of areas of a cotton field heavily (A) and lightly (B) infested with lance nematodes. C-E) Photomicrographs of lance nematode injury in cotton roots: C) lance nematode in cortical tissue (see arrow); D) nematode feeding in phloem tissue; E) membrane across penetrated cortical cell (see arrow). Photomicrographs 380×.

A
B
C
D

greenish or reddish. Undamaged phloem stained green. Twice nematodes were found with their stylets in phloem parenchyma cells.

In several roots, abnormal division of phloem parenchyma occurred in response to nematode damage as if to form cork tissue (Fig. 2, C, D). The cells divided tangentially, and several divisions occurred. Some cells showed 2 tangential divisions, whereas others showed only 1.

5) *Xylem damage.*—The lance nematode also punctured xylem elements, but not nearly as often as phloem. Xylem in close proximity to parasitized phloem was damaged, even though no xylem elements had been attacked. Xylem was as extensively damaged as phloem. In a xylem group close to damaged phloem, only 1 or 2 xylem vessels nearest the phloem were damaged, or entire groups were affected. The amount of affected xylem was related to the extent of nematode damage and to age of the damage. Nematodes had not punctured all the way through the vascular tissue in any of the roots sectioned.

Tylose production occurred in xylem cells associated with injury. In none of the sections examined were tyloses present in nonaffected cells. The amount of tyloses present was directly related to the age of the injury (Fig. 2, B).

DISCUSSION AND CONCLUSIONS. — The isolation of lance nematodes in large numbers from the soil around debilitated cotton plants in the field indicated that these nematodes play a major role in causing this condition. The severe stunting and defoliation of cotton plants in areas of high nematode infestation, however, undoubtedly was accentuated by the dry condition of the soil. It is natural for plants with mutilated root systems to be more seriously affected during dry periods than plants whose roots are not damaged by nematodes. Plants growing in areas of low lance nematode infestation were not seriously affected by the dry season. Nematodes of the genera *Helicotylenchus*, *Pratylenchus*, and *Meloidogyne* also were recovered, but probably contributed very little to the stunted condition of the plants because of the small numbers present.

Greenhouse studies indicated that cotton plants when supplied with sufficient moisture and fertility can tolerate rather high populations of the lance nematode. Only slight stunting occurred under greenhouse conditions with populations comparable to those found in the field where severe stunting was observed.

Although the lance nematode feeds readily on the epidermis and cortex, phloem appears to be the preferred tissue. Occasionally a few xylem cells were punctured. Food materials, principally sugars, formed by the leaves are translocated through the phloem to the roots for storage and utilization. These materials may attract lance nematodes to the phloem.

In cotton, the lance nematode feeds primarily as an endoparasite and large numbers may enter the roots. Migration through the root forms a passageway by which secondary pathogens and saprophytic organisms may gain entrance to root tissue and add to the damage. Thus, lance nematodes might be expected to increase the incidence of other root diseases of cotton, such as Fusarium wilt.

In addition to those cells upon which the nematode feeds directly, many cells in the cortex, endodermis, and vascular tissue that are out of reach of the nematode's stylet become devoid of contents. Death of these cells could be due to one or a combination of any or all of the following factors: substances secreted by the nematodes, secondary organisms that gained entrance to the root as the result of the nematode injury, and decomposition products of adjacent killed cells. It is known that some plant parasitic nematodes secrete substances into cells that partially digest cell contents before they are sucked into the nematode.

Damage is more extensive in the vascular tissue than in the cortex. In the vascular tissue, the more rapid translocation probably carries the injurious materials much farther than in the cortex. Damage may extend several millimeters in both directions from the feeding point in the xylem and phloem, whereas in the cortex and endodermis it is restricted to a few cells surrounding the nematode. The lower rate of translocation in the cortex and endodermis may account for the lesser damage in these tissues. Also, there is more concentrated feeding on the vascular tissues.

The staining characteristics of injured cells present another interesting phenomenon. The red staining associated with cells fed upon directly by the nematode and the nature of its spread to surrounding nonpunctured cells suggest nematode feeding secretions as the cause of this condition. Other possibilities are that decomposition products of dead penetrated cells or metabolic products of secondary organisms allowed entrance to the root by nematode wounding may be responsible for this staining reaction. The material that stained red must be basic in reaction because of its affinity for safranin, an acidic dye. By these staining reactions, lance nematode injury in cotton roots may be located easily.

Cell membranes apparently are formed in many cortical cells and in nearly all endodermal and vascular cells soon after penetration by nematodes. This membrane lacks the characteristics of a true cell wall.

FIG. 2. Photomicrographs of lance nematode injury in cotton roots: A) endodermal damage; B) tyloses in xylem elements (see arrow); C) abnormal cell division in phloem parenchyma (see arrows); D) abnormal cell division of phloem parenchyma, in cross section (see arrows). Photomicrographs 380×.

It is of a thick viscid character, lacks birefringence, and stains nearly black. The chemical nature of this membrane is not known.

In older damage, tangential divisions occurred in phloem parenchyma in the vicinity of injured tissue. In some areas many divisions had occurred, whereas in others only 1 or 2 were apparent. This seems to be a host reaction to nematode injury. It is not known whether this division is purely a mechanical response to injury or whether it is due to nematode secretions that stimulate the division.

Sometimes tyloses were formed in normal xylem elements of cotton, but not in great abundance. Around the nematode feeding area, however, tyloses were formed in great profusion and completely filled many cells in affected xylem. These tyloses stained as deeply red as the rest of the cell in which they were present. Tylose production evidently was stimulated by lance nematode feeding. Under conditions of heavy nematode infestation, decreased translocation brought about by the plugging of injured xylem elements by tyloses must be partly responsible for the stunting of infested cotton plants.

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COMPARATIVE CONTROL OF BUCKEYE ROT OF TOMATO BY VARIOUS FUNGICIDES [1]

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SUMMARY

Buckeye rot of tomato (caused by *Phytophthora* spp.) occurs only sporadically in Ohio. In an experimental planting of tomatoes being sprayed for the control of anthracnose fruit rot at Wooster in 1955, the best control of buckeye rot was furnished by captan, followed by maneb. Ziram gave very poor control, but when half of it was replaced with a fixed copper in a tank-mix formulation, markedly increased control of the disease resulted. A tank-mix formulation of maneb was much less effective than was a wettable powder preparation. The varieties Pennhart, Bounty, and Jefferson were much more susceptible to infection than were Jubilee, Victor, and Sioux.

Buckeye rot of tomatoes has been variously ascribed to at least 4 species of *Phytophthora*, apparently depending upon the geographical area in which it occurred (2, 3, 4, 5). The disease appears sporadically and then only in localized areas in Ohio. Almost every year, however, it is reported from some field, garden, or associated group of fields, where in numerous instances it causes a considerable reduction in yield. It is found rather frequently on staked tomatoes in the Marietta area, even though staking often is mentioned as a method of control of the disease. When it occurs on the fruits of staked plants, it usually is found in low portions of a field and/or in drainage channels where water stands or flows for brief periods following heavy or flash rain storms. The same is true for its occurrence on fruits in contact with the soil on unstaked plants in Ohio and elsewhere (2, 3, 4, 5).

The disease has caused economic loss in the vicinity of the Experiment Station at Wooster at least twice during the last 10 years, once in 1949 and again in 1955, and it has been found in experimental plots during at least 2 other years. It became quite severe for a short period in early August of 1955, following heavy rains during July, in a planting of 12 tomato varieties being sprayed with several different fungicides, and/or formulations of the same, for the control of anthracnose fruit rot. Fruits attacked by buckeye rot were first observed during harvest on August 3, just as the earliest varieties (Fireball, Earliana, and Victor) were beginning to ripen. The infection sequence persisted for about 4 weeks during which time the fruits were wetted frequently by rains heavy enough to cause temporary flooding of the soil surface with which many of them were in contact.

On August 10, as many as 10 or 20 of the green fruits of all sizes were infected on some of the plants of certain of the varieties included in the experiment. Since there was an obvious difference in the number of rotted fruits in the differently treated (sprayed) plots, all diseased fruits were removed and counted on that date during the harvest operation. A few more rotted fruits had appeared by August 24, and a second count was made at that time. The control of the disease by different fungicides and the differences in varietal susceptibility to infection are shown by the data in Tables 1 and 2, respectively. In this planting, the maximum number of diseased fruits in any 1 plot of 9 plants occurred in an untreated plot of the Pennhart variety, from which 163 specimens were removed on August 10. The same variety yielded only 3 diseased fruits from the plants in another plot that had been sprayed with captan. Control of the disease by captan was not this effective on all varieties, but the average control by captan was significantly better than that given by any other treatment applied for the first time on the same date (July 11). Dithane M-22 (70 per cent maneb) applied on July 1 was not significantly poorer than captan, but this seeming advantage of applying the first spray earlier in the season was not maintained with an application made on June 21 (Treatment 3). Only Treatment 11 was significantly poorer than all others. Ziram, in Treatments 8 and 10, gave comparatively poor control of the disease.

Felix (2) found that 2 fixed coppers, C-O-C-S (basic copper chloride and basic copper sulfate; 55 per cent Cu) and Tri-Basic, reduced buckeye rot by 80–90 per cent in an experiment conducted in Tennessee in 1949. Neither of these materials was selected for use as the sole fungicide in any treatment in the present experiment, since copper-containing compounds commonly give very little control of anthracnose. Tri-Basic was used in a tank-mix formulation with maneb and ziram (Treatments 7 and 9, respectively). The mixtures controlled the disease better than did either of the 2 organic compounds used alone (Treatments 4 and 8, respectively). The increase in control when copper was added to maneb was slight, but the addition of copper markedly increased the control with ziram.

The addition of an adhesive in the form of K704 (a polyacrylate polymer) increased the effectiveness of maneb slightly. A tank-mix formulation of ziram (Treatment 10) controlled the disease better than did Zerlate, a wettable-powder formulation of ziram. On the other hand, a tank-mix preparation of maneb

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TABLE 1.—*Incidence and control of buckeye rot in a 12-variety planting of tomatoes at Wooster in 1955*

Treatments[a]	Lb./100 gal.	Total number of fruits diseased on 108 plants	Percentage diseased	Percentage control when check = 0
1. None		1276	31.3	0.0
2. Dithane M-22	2	555	10.1	56.5
3. Dithane M-22	2	450	8.0	64.9
4. Dithane M-22	2	558	10.4	56.3
5. Dithane M-22 + K704	2, ½	485	8.8	62.0
6. Dithane M-22 + Zerlate	1, 1	737	13.4	42.4
7. Dithane M-22 + Tri-Basic	1, 2	489	9.3	61.7
8. Zerlate	2	990	19.6	22.5
9. Zerlate + Tri-Basic	1, 2	539	10.0	57.7
10. SDD + $ZnSO_4$	2, 1	636	14.3	50.0
11. Dithane D-14 + $MnSO_4$	4,1	1036	25.6	18.8
12. Captan 50-W	3	186	3.3	85.5
LSD at 5 per cent level			5.2	

[a] First spray in Treatment 2 was applied on June 21; that on Treatment 3 was applied on July 1. All others were started on July 11 and were continued at 10-day intervals until September 19. Dithane M-22 is 70 per cent maneb (manganese ethylenebis[dithiocarbamate]); K704 is a polyacrylate polymer; Zerlate is 76 per cent ziram (zinc dimethyldithiocarbamate); Tri-Basic is basic copper sulfate ($CuSO_4 \cdot 3\ Cu(OH)_2$) containing 53 per cent Cu; SDD is 40 per cent sodium dimethyldithiocarbamate; Dithane D-14 is 19 per cent nabam (disodium ethylenebis[dithiocarbamate]); and Captan 50-W is 50 per cent captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide).

(Treatment 11) was much less effective than was Dithane M-22 (Treatment 4), a wettable powder containing maneb. This difference in the comparative performances of the wettable-powder and tank-mix formulations of ziram and maneb occurs in the control of other diseases, such as early blight of potato and of tomato and tomato anthracnose (6).

The matter of relative susceptibility to buckeye rot of the different varieties included in this experiment is not of great importance, since the disease occurs in epidemic form so seldom in Ohio, but it is interesting to observe how widely the varieties differed in the number (percentage) of fruits that became infected. The varieties most susceptible to buckeye rot, such as Pennhart, Bounty, Jefferson, and Fordhook Hybrid, were not necessarily the same as those that showed the highest percentages of anthracnose fruit rot later in the season (Earliana, Victor, Pennhart, and Fordhook Hybrid).

TABLE 2.—*Comparative susceptibility of 12 tomato varieties to buckeye rot*

Variety	Percentage diseased
Pennhart	15.1
Bounty	13.2
Jefferson	12.3
Fordhook Hybrid	11.4
Grothen's Globe	10.0
Earliana	9.7
Sunnybrook Hybrid	9.6
Fireball	9.2
Clark's Spec. Early	8.8
Jubilee	6.5
Victor	5.9
Sioux	4.6

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HEREDITARY RESISTANCE TO TOBACCO-MOSAIC VIRUS IN TOMATO [1]

James M. Walter

SUMMARY

Effective resistance to tobacco-mosaic virus, *Marmor tabaci* Holmes, has been a prime objective in the breeding of disease-resistant tomatoes in Florida since 1942. Semiprimitive selections in advanced generations from a 3-way cross ((Rutgers × *Lycopersicon hirsutum* Humb. & Bonpl., P.I. 126445) with Indiana Baltimore) demonstrated homozygosity for effective resistance to a combination of green and yellow strains of the virus. Slow but definite progress has been made in transferring this resistance to improved larger-fruited tomato types and in combining it with resistances to other important pathogens.

Resistance to tobacco-mosaic virus is nonsymptomatic tolerance. Its usefulness is nullified by superimposition of other viruses such as tobacco etch virus and potato viruses X and Y. Inheritance of resistance was studied on large, heavily inoculated F_2 populations from crosses between resistant and susceptible stocks deemed most likely to lead to the practical objective. The ratios observed closely approximate 63 susceptible to 1 resistant, indicating that the parents differ with respect to reaction to tobacco-mosaic virus by 3 recessive genes and that the resistance is unlike any of the types recently described by other workers.

Those who produce tomatoes (*Lycopersicon esculentum* Mill.) on an intensive scale and strive to market a high-quality product have been keenly aware for many years of the need for some means of controlling tobacco-mosaic virus, *Marmor tabaci* Holmes. (Hereafter in this article, TMV is used as an abbreviation of tobacco-mosaic virus.) This virus is present in several common weeds in practically all areas where tobacco and tomato are grown, and it remains viable for many years in home-cured tobacco and in numerous manufactured tobaccos. These primary sources of inoculum pose a problem against which very few growers manage to arrange satisfactory defenses.

The virus is readily transmitted from diseased to healthy plants by the handling required in the culture of tomatoes grown on supports. The reductions in yield and quality of tomatoes due to TMV are much more important than most people interested in the crop realize. The reduction in gross yield of susceptible plants is not great if infection does not occur until several fruits are well developed, but any fruit that is less than ¾ in. in diameter at the time of infection is likely to be curtailed to approximately ⅔ normal size and is almost certain to be markedly inferior to a normal fruit in color, texture, and flavor. Because of these facts, an effective hereditary resistance to TMV has been sought by numerous workers (1, 2, 3, 5, 8) striving in recent years to improve tomato varieties.

In the tomato-breeding program at the Gulf Coast Experiment Station, resistance to TMV rates next to resistance to Fusarium wilt (incited by *Fusarium oxysporium* f. *lycopersici* (Sacc.) Snyd. & Hans.) in the list of objectives. Breeding for resistance to TMV was begun at this station in 1942 by Dr. J. R. Beckenbach, who grew the F_2 generation of a 3-way cross ((Rutgers × *Lycopersicon hirsutum*, P.I. 126445) × Indiana Baltimore) from seed supplied by Dr. Ralph E. Lincoln of Purdue University and propagated a number of selections. During the period 1943–1946, the work was continued and increased in scale by Dr. A. L. Harrison, who acquired breeding stocks in addition to those he brought with him. A long list of accessions and reasonably complete records concerning them were available to the writer when he began work on the project in January, 1947.

TESTING PROCEDURE AND KIND OF INOCULUM USED.—Dr. Harrison's records made it apparent that breeding for resistance to TMV in tomato involves many difficulties: the erratic performance of some of his selections could not be satisfactorily explained. His notes suggested the advisability of testing the numerous accessions with an identified strain of TMV because of the possibility that differences among strains of the virus might account for some of the observed irregularities. Dr. S. P. Doolittle kindly supplied for this purpose Johnson's type strain (American type culture No. 2) of green tobacco-mosaic virus. After a few weeks of testing of susceptible, segregating, and resistant stocks heavily inoculated with this strain of the virus, however, it was concluded that the symptoms in tomato plants were too vague to serve as a basis for culling susceptible seedlings from a segregating population with any satisfactory degree of assurance.

Unidentified collections of yellow tobacco-mosaic virus obtained from diseased tomatoes in nearby fields were available. One[2] of these collections that had met the readily determined criteria for TMV (production

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The aid of R. A. Conover, D. G. A. Kelbert, N. C. Hayslip, and J. C. Noonan in evaluating the horticultural adaptation of advanced selections is gratefully acknowledged. These staff members of the Florida Agricultural Experiment Stations are co-leaders in the tomato-breeding project.

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[2] The writer is indebted to Dr. W. D. Valleau for checking the identification of this collection. He pronounced it "just one of the many strains of yellow TMV already described."

of only local necrosis on leaves of Kentucky 56 (NN Burley) tobacco (*Nicotiana tabacum* L.) and resistance to heat and drying) was used for reinoculation of half the plants in each population in a trial in which the green strain had failed to produce distinct symptoms. Some of the reinoculated plants soon showed readily discernible symptoms; others appeared resistant. Another series of the same tomato stocks was immediately inoculated for a comparison of the symptoms produced by the green strain, by the yellow strain, and by the 2 strains in combination (juices mixed in equal parts). The results clearly suggested that the combination would give the most distinct symptoms. Moreover, it seemed possible that a resistance applying to both green and yellow strains might be more generally effective than a resistance applying to either one of the types. For these reasons, the combination of the 2 virus strains was put into stock culture in Turkish tobacco and subsequently has been propagated in this form to supply all the infectious juice needed for the work reported herein.

The inoculation of populations of tomato seedlings with TMV was started as soon as the plants began unfolding the first true leaves. This was done by the technique described by Richards and Munger (9), which allows a very great saving of time. From 6–10 days later, the plants were again inoculated by the same method. Roguing symptomatic plants was begun 10–14 days after the first inoculation (Fig. 1) and was repeated at weekly intervals. In the course of roguing, time was taken to rub the stem of each nonsymptomatic plant hard enough to break a few hairs. Normally, a population of plants that had to be transplanted in the field by the time the larger individuals were 1 ft. tall had received, prior to transplanting, 2 applications of TMV by Richards and Munger's technique and 2 by hand during the 2 roguings of symptomatic plants.

It was the experience of Harrison and has continued true in the writer's experience that no high percentage of susceptible plants can be culled from a segregating population by the time the plants reach the maximum size for satisfactory transplanting. Instead, many of the segregates are slow to show symptoms. Quite often it happens that many of the susceptible plants do not show mosaic symptoms until they have recovered from transplanting and begin to make rapid growth in the field. This delay adds considerably to the land and labor needed for the project, but to date no means of avoiding the expense of transplanting seedlings of questionable reaction has been devised.

DESCRIPTION OF THE RESISTANCE.—Resistance was

FIG. 1. Tomato seedlings at stage for roguing, 2 weeks following first inoculation with tobacco mosaic virus. From left to right: first row, resistant parent; second row, susceptible parent; third and fourth rows, F_2. In the last 2 rows, approximately ⅔ of the plants were clearly symptomatic and were culled on the following day. Photograph by D. C. A. Kelbert.

first convincingly demonstrated to the writer in semiprimitive selections in the F_8 to F_{10} generations from the 3-way cross ((Rutgers × *L. hirsutum*) × Indiana Baltimore) previously mentioned. By the end of 1948, performance through repeated tests and through 2 or more generations had made it obvious that a few selections of this origin were uniform for resistance and that resistance had been transferrred to some of the progeny of crosses between these semiprimitive selections and large-fruited, wilt-resistant stocks (10).

Transmission tests regularly demonstrated that TMV is present in the terminal-growth tissue of nonsymptomatic resistant plants that previously have been thoroughly inoculated with TMV. In these transmission tests, the bud leaves used as sources of the juice applied to the test plants of NN tobacco were separated from the inoculated portions of the source plants by at least 1 ft. of intervening growth. In view of the evidence that thoroughly inoculated plants contain TMV, the resistance is perhaps best described as nonsymptomatic tolerance.

It was soon apparent that this resistance to TMV may be nullified if a virus of another type is superimposed. Plants of TMV-resistant selections that have been inoculated grow without symptoms despite the fact that TMV is present in them, but after inoculation with a virus of another type, such as tobacco etch virus (*Marmor erodens* Holmes), they soon show typical symptoms of tobacco mosaic. Other viruses found to nullify this resistance to TMV are potato virus X (*Annulus dubius* Holmes) and potato virus Y (*Marmor upsilon* Holmes).

Despite these short-comings, this resistance to TMV is very effective in preventing the unfavorable effects of tobacco mosaic on both leaves and fruits of tomato. Seed supplies of lines fixed for resistance have been available, beginning in 1949, for division with other workers and for numerous field trials. Whether tested in field or greenhouse, in winter or summer, in Florida or a northern state, the resistance has thus far proved highly effective against TMV. In contrast with this, stocks furnished the writer by Dr. W. A. Frazier (then of Hawaii Agr. Expt. Station) and by Dr. Douglas Bain of Mississippi State College have proved susceptible to TMV when subjected to the routine inoculation test at this station.

INHERITANCE OF RESISTANCE.—It has been obvious from the beginning of these tests that inheritance of the above-described resistance to TMV is not simple. During the past 18 crop seasons, the number of plants of segregating populations set in the field has averaged near 5000. Any season that has yielded 6 selections that proved uniform for resistance during the next 2 generations has been counted successful. Of course, losses of plants to flood, soil fungi, other viruses, etc., have been heavy nearly every season.

Advancement of resistance to commercially useful varieties always has been considered the primary objective. Consequently, the limited facilities for production of seed supplies have been used for those new hybrids believed most likely to lead to this objective. It was realized, of course, that an understanding of the inheritance of the resistance might allow simplification of the breeding program. At every opportunity that has arisen for the past 7 years, tests have been run for the sole purpose of gathering data on the mode of inheritance. Only rarely, however, has a test yielded data that could be considered reliable. The upsetting factors have been many, including damage to plants by excessive doses of modern insecticides. DDT, DDD, and methoxychlor were found to produce chlorotic and stunting effects much like those of TMV and caused the discard of several trials.

The most reliable test on inheritance of resistance that has been carried out to date involved 1095 plants of the F_2 from the cross 54-1-2 CAStMW × CAStW 131—12, plus 1960 plants of the reciprocal cross. The former parent, of course had proved to be uniform for resistance. The latter parent has a record of high susceptibility but is a horticulturally attractive selection. The F_1 from this cross was susceptible. The F_2 seedlings were kept under observation for 90 days following the first inoculation and were inoculated 3 more times during the first 50 days of the period. At the end of 90 days, 3007 of the 3055 plants had developed symptoms of TMV. This observed ratio of 62.65 susceptible to 1 resistant is so near the theoretical ratio of 63 to 1 that it is very highly suggestive that the parents differ by 3 recessive genes with respect to their reactions to TMV.

Another test involved an F_2 closely related to that just described. The susceptible parent was CAStW 131—12, and the resistant parent (55-3-2-Bk CAStMW) was derived from the reciprocal of the cross that produced 54-1-2 CAStMW. The F_2 seedlings were first inoculated on June 30 and then kept under observation for the next 122 days. At the end of this time, 124 plants of a total population of 8069 remained nonsymptomatic. This observed ratio of 64.07 susceptible to 1 resistant is again very close to the 63:1 ratio and is based on a sufficiently large population to seem reasonably conclusive on the point that the parents differ by 3 recessive genes.

It seems almost certain, in view of the complex breeding of the tomato stocks now in use, that projection of the foregoing results on inheritance to many other crosses would be subject to error. Though it is considered likely that *Lycopersicon hirsutum* (P. I. 126445) was the source of all the genes controlling the resistance, it is possible that *L. pimpinellifolium* (Jusl.) Mill. or some other tomato-type in the involved pedigree contributed important germ plasm.

From this evidence, it appears probable that this resistance is different from those described by Frazier and coworkers (4, 8), Holmes (6, 7), and Watson et al (11). Although it is possible that these workers have been dealing with resistances that are a part of the resistance here described, their statements indicating that resistance is dominant to susceptibility cast some doubt on even this possibility.

Present status of practical breeding program.—As indicated previously, progress toward the goal of commercially acceptable tomatoes inherently resistant to TMV has been slow. In view of the long odds against the selection of segregates homozygous for resistance to TMV, it has been necessary to select first for resistance and to trust to chance that some resistant selections will have other requisite traits. One of the discouraging experiences to date has been that nearly all selections that have bred true for resistance have been late maturing and defective in quality of the fruits. Several of these selections have proved to have definitely objectionable fruit flavor. In contrast, it has not been difficult to obtain selections that have resistance to Fusarium wilt, to gray leaf spot (incited by *Stemphylium solani* Weber), and to leaf mold (incited by *Cladosporium fulvum* Cke.) in combination with the resistance to TMV.

Relatively steady progress is being made and it now appears that resistance to TMV can be established in commercially acceptable tomato-types within the next few years. Several stocks under observation and selection in the F_3 to F_5 generations appear very promising. At present, however, not one of the more advanced selections on hand shows the dependable yielding ability required in a new variety. Those that have good quality and flavor of fruits are late maturing and usually are low yielding. Those that bear early and heavy are defective in 2 or more important features of the fruits, such as sensitivity to blossom-end rot and to growth cracks. Recently, progress has been hampered by rapid spread of potato virus Y in the plantings. Although it is clear that such difficulties will give further trouble and that there is much yet to be learned, there are reasons to believe that new varieties carrying this effective resistance should become available within the next 5 to 10 years.

Seed of a number of selections fixed for this resistance to TMV plus resistances to some other important pathogens are available to interested investigators.

GULF COAST EXPERIMENT STATION
BRADENTON, FLORIDA

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COMBINATION OF RESISTANCES TO TOBACCO-ETCH AND TOBACCO-MOSAIC VIRUSES IN TOMATO BREEDING STOCKS [1]

James M. Walter

SUMMARY

Discovery that the resistance to tobacco-mosaic virus in tomato is nullified by tobacco-etch virus, *Marmor erodens* Holmes, stimulated search for tomato breeding stocks resistant to etch virus. P.I. 183692, an attractive form of *Lycopersicon esculentum* Mill. from Turkey, was found to be resistant. Mating P.I. 183692 with CStMW18, the most advanced stock resistant to tobacco-mosaic virus then available, resulted in a few selections that proved to have the resistances to etch virus and tobacco-mosaic virus in combination. The resistances are effective whether the viruses are applied separately or in combination. Younger stocks from matings intended to transfer these resistances to improved tomatoes with resistances to fungus pathogens are showing promise.

Preliminary studies suggest that resistance to etch virus is actually immunity and that 1 recessive gene determines its inheritance.

The broad objective of the tomato-breeding project of the Florida Agricultural Experiment Station is to combine, in a few generally useful varieties, effective hereditary resistances to the several pathogens important on the many acres of tomatoes grown in the State. The scope of the project and the dynamic nature of the multifaceted industry require frequent changes in the thinking concerning the relative importance of the several diseases. For example, recent progress in marketing tomatoes harvested in the pink stage calls for a higher ranking of diseases due to mechanically transmitted viruses.

The tobacco-etch virus, *Marmor erodens* Holmes, on tomato is an example of a relatively minor pathogen that quickly assumed sufficient importance to merit attention in the tomato-breeding program. The mild mosaic of tomato due to this virus increased rapidly enough in the Manatee-Ruskin area during the 1948-50 period to cause concern; but the effects were not spectacular, and it is possible that a severe frost would have the beneficial effect of cleansing the area of this virus. When it became apparent, however, that incursions by tobacco-etch virus (TEV is used as an abbreviation for this name hereafter in this paper) nullified the resistance to tobacco-mosaic virus (TMV is used as an abbreviation for this name hereafter in this article) as reported previously,[2] it seemed highly desirable to determine whether resistance to TEV could be found in tomato and effectively combined with resistance to TMV.

The only reference that could be found concerning resistance to TEV among *Lycopersicon* species occurs in a publication[3] by Holmes, who was unable to supply a stock that could be tried in the breeding program. It was known that plant explorers working for the U. S. Department of Agriculture had recently sent numerous collections of *Lycopersicon* from other countries to plant introduction stations in the U.S.A. Through Dr. Edwin James, Regional Plant Introduction Station, Experiment, Georgia, and Dr. M. M. Hoover, Regional Plant Introduction Station, Ames, Iowa, arrangements were made to obtain seed samples of these P.I. accessions for the purpose of testing their reactions to TEV. Testing was begun in the autumn of 1950 and continued until 341 accessions had been tried. P.I. 183692 was found resistant to repeated inoculation with TEV during the winter of 1951–52, and this result was corroborated by further trials during the following winters.[4]

DESCRIPTION OF P.I. 183692.—P.I. 183692 (*Lycopersicum esculentum* var. Scarlentawen), which was collected at the Mayislar Cotton Testing Farm, Eskischir, Turkey, is an attractive type that possibly ranks as a standard variety or as a horticulturally useful type in the region in which it was collected. It produces numerous 3-locule fruits that are smooth and deep, and it is medium in maturity (Fig. 1). It has proved very susceptible to every pathogen except TEV to which it has been exposed at the Gulf Coast Experiment Station.

BREEDING RESULTS WITH P.I. 183692.—In the spring of 1952, P.I. 183692 was used as the pollen parent for a cross with CStMW 18-1-1-1-Bk-Bk, the latter being the best available stock known to be fixed for resistance to TMV. Because CStMW 18-1-1-1-Bk-Bk is late and normally not highly yielding, it was considered that the cross would be helpful in leading toward the horticultural characters needed in tomatoes resistant to TMV, whether or not the resistances to TEV and TMV could be effectively combined. Seedlings of the F_1 from this cross, labelled 1 CEStMW, proved suscepti-

[1] Accepted for publication April 12, 1956.
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[2] Walter, J. M. 1956. Hereditary resistance to tobacco-mosaic virus in tomato. Phytopathology 46: 513-516.

[3] Holmes, F. O. 1946. A comparison of the experimental host ranges of tobacco-etch and tobacco-mosaic viruses. Phytopathology 36: 643-659.

[4] Alexander, L. J., and M. M. Hoover. 1955. Disease resistance in wild species of tomato. North Central Regional Publication 51. Ohio Agr. Exp. Sta. Res. Bul. 752. (Cf. p. 23).

FIG. 1. Mature plants of P. I. 183692. Photograph by D. G. A. Kelbert.

ble to both viruses in separate trials during the following year. Since further testing of P.I. 183692 confirmed the evidence that it possessed resistance to TEV, a goodly supply of seed for the F_2 generation of 1 CEStMW was produced. In the summer of 1953, a large population of the F_2 generation was grown and tested for reaction to TMV. During the autumn, 12 selections for resistance to TMV in combination with desirable fruiting habit and resistance to gray leaf spot (incited by *Stemphylium solani* Weber) were made from this F_2 population. When these 12 selections were tested in the F_3 and F_4 generations for reactions to TMV and TEV (separately), 5 were found to be breeding true for TMV resistance and 2 of the 5 proved to be uniform for resistance to TEV as well. Since performance of the F_3 plants of the 2 families resistant to TEV made it seem very doubtful that either could lead to a horticulturally acceptable selection, the plants that were judged to be the best were used immediately for crosses with the top-ranking horticultural types on hand. This was done in the spring of 1954, and the first 3 generations from the most promising of these crosses were grown during the next year. The tests of reactions of the F_3 selections indicate that a few of them, which offer some promise of being highly productive, are homozygous for resistance to

TABLE 1.—*Reaction of F_4 populations of tomato crosses to tobacco-etch virus*

Stock designation	Parentage	Number with indicated reaction to TEV	
		Susceptible	Resistant
1 CEStMW	CSTWM 18 × P. I. 183692	344	91
17 ACEMStW	Manalucie × 1-10-3 CEStMW	269	97
4 ACEFMPRStW	1-5 ACFMPRStW × 1-10-3 CEStMW	303	106
Totals		916	294
Ratio of number susceptible to number resistant		3.1	

both TEV and TMV. These results must be corroborated by results on the F_4 generation before being accepted as reliable. This breeding record shows clearly that there has been no difficulty in combining the resistances to the 2 viruses.

During the winter of 1954–1955, seedling populations of F_5 selection 1-10-3-2 CEStMW were subjected to replicated testing of their reactions to TEV and TMV in combination. In separate inoculation tests, this stock had proved uniform for resistance to each virus; it likewise proved resistant when inoculated with the 2 viruses in combination and when inoculated first with TMV and later with TEV.

To date, only 3 F_2 populations of approximately 400 plants each have been tested in a manner that would provide data on the inheritance of resistance to TEV. These 3 populations and the numbers of plants, by classes for reaction to TEV, are given in Table 1.

These data so closely approximate the ratio of 3 susceptible to 1 resistant that they have led to the tentative conclusion that the resistance to TEV is controlled by a single recessive gene. The slight preponderance of susceptible plants in 1 CEStMW seems reasonable because the seed were not produced under isolation and circumstances were favorable for natural cross-pollination.

THE RESISTANCE TO TEV MAY BE IMMUNITY.—Transmission trials have been made to test plants of tabasco pepper, *Capsicum frutescens* L., from F_2 plants of 17 ACEMStW that had been heavily inoculated with TEV. Juice from bud leaves of 5 segregates that had remained apparently healthy, despite 3 inoculations with TEV, was rubbed quickly on leaves of tabasco peppers. None of these peppers developed symtoms of TEV. Similar transmissions to tabasco peppers were made from symptomatic tomatoes of the same population. All peppers of this lot developed a blackened collar and quick decline of the entire plant above the black ring, the characteristic reaction of tabasco pepper to TEV[5]. For a methods-control series, leaves of tabasco peppers were rubbed with tap water after the hands

[5] Greenleaf, W. H. 1953. Effects of tobacco-etch virus on peppers (Capsicum sp.). Phytopathology 43: 564-570.

had been cleansed as in preparation for each transmission effort just described. All plants of this lot remained healthy.

This preliminary evidence suggests that the virus is not present in the tomato segregates that show no symptoms, and it thus suggests that the resistance may be immunity.

PRESENT STATUS OF BREEDING PROGRAM. Several selections that have been found to possess, in combination, resistance to TMV, TEV, *Stemphylium solani*, and *Fusarium oxysporum f. lycopersici* (Sacc.) Snyd. & Hans. are now in the F_7 generation. Field trials have shown that, in the F_5 and F_6 generations, some of these selections are very prolific but have defects of the fruits, such as roughness, susceptibility to radial cracks, poor flavor, and unattractive internal coloring —defects that would make them unacceptable as commercial varieties. Preliminary results indicate that the resistances to TMV and TEV are in combination in younger selections, representing generations F_3 to F_5. These younger stocks require further testing and refining by selection, but they should allow good opportunities for improvement in features of fruits as well as for combinations of resistances to other pathogens.

Seed of these new tomato types are available for any one who wishes to use them either for breeding stocks or for studies on the resistances.

GULF COAST EXPERIMENT STATION
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ASSAYS FOR RESISTANCE TO CUCUMBER MOSAIC IN THE PICKLING CUCUMBER [1]

J. B. Sinclair[2] and J. C. Walker

SUMMARY

Cucumber mosaic on cucumber plants of susceptible and resistant pickling varieties developed much more rapidly and severely in the greenhouse than in the field. The rate of disease development in the greenhouse increased with temperature from 16° up to 28°C. At 16° and 20°, resistant and susceptible plants differed little in their reaction. At 24° and 28°, the difference widened during spring and summer days, but the difference was small even at these temperatures during midwinter. At 24° and 28°, resistant plants tended to outgrow the shock phase of the disease and to show less expression of mottle on young leaves. The ratio of height of noninoculated to that of inoculated plants was generally somewhat higher under those conditions. A decided reduction in total yield of fruits of susceptible varieties as compared with that of resistant varieties commonly occurred when seedlings were inoculated at about the third-leaf stage in the field. In some cases, however, there was no significant difference in yield of resistant and susceptible varieties. In all cases, the percentage of mottled fruits in susceptible varieties increased steadily during the harvest season; in resistant varieties, the percentage of mottled fruits remained much lower and the mottling was much less severe. The percentage of mottled fruits, therefore, was considered the most reliable final criterion in judging the relative resistance of varieties and of individual plants. Greenhouse assays may be used as a supplementary criterion for measuring resistance, but they should be carried out under relatively high light intensities and at temperatures of 24°–28°C. Even under those conditions the difference in reaction of resistant and susceptible varieties may be small, and this assay can only be used reliably as a supplement to field tests.

One of the problems that arises in the development of cucumber (*Cucumis sativus* L.) varieties resistant to cucumber mosaic virus is that of proper conditions under which assays of progenies for resistance may best be conducted. Resistant varieties now in use in the United States are derived from crosses of Oriental resistant varieties with commercial varieties. In all of the resistant lines so far secured, cucumber mosaic virus increases readily as shown by Sill and Walker,[3] but under field conditions the host is capable of withstanding the effects of the virus and of producing an acceptable crop both as to quantity and quality. The purpose of the study reported herein was to compare the expression of resistance in the field with that under controlled conditions in the greenhouse in order to determine whether a standard set of greenhouse conditions for assay could be found. Such a standard

[1] Accepted for publication April 24, 1956.
This investigation was supported by a grant from H. J. Heinz Company.

[2] Present address: Assistant Plant Pathologist, Louisiana State University, Baton Rouge, Louisiana.

[3] Sill, W. H., Jr., and J. C. Walker. 1952. A virus inhibitor in cucumber in relation to mosaic resistance. Phytopathology 42: 349-352.

would permit the evaluation of resistance at various times of the year, would shorten the time required for assay, and would permit the assay to be conducted with different strains of cucumber mosaic virus as well as of other viruses affecting cucurbits without danger of contamination by insect vectors.

FIELD EXPERIMENTS.—The first results to be presented are those from field trials of resistant and susceptible varieties at Madison, Wisconsin, during the summers of 1951, 1952, 1953, and 1954. The seed was sown during the last half of May according to the usual commercial procedure. In 1954, an additional late planting was made in late June. Plants were inoculated by rubbing cotyledons and first leaves with an extract from infected plants of *Nicotiana glutinosa* L. after the plant parts to be inoculated had been sprinkled with Carborundum. A common field strain of cucumber mosaic virus was used throughout the experiments. Pickling varieties were used, and the fruits were picked 3 times weekly beginning soon after July 15. National Pickling (NP) was used throughout as the susceptible variety and Ohio MR17 (MR17) as the resistant variety. Other varieties were used in some cases as indicated. In each year, symptoms of the disease in the form of mottled leaves and suppression of stem elongation in the susceptible variety were negligible until 4–6 weeks after inoculation. In the 1951, 1952, and 1954 trials, stunting of vines in the susceptible variety, in comparison with the resistant variety, became increasingly evident after the second week of harvest. It was least pronounced in the early planting of 1954. In the 1953 planting, however, the 2 varieties differed little in growth. The end results of these trials are reflected in the total yields (Table 1). Differences in 1953 and in the early planting of 1954 were not statistically significant. The trials of 4 years show that yield is not a completely reliable measure of resistance.

A second criterion for measuring resistance was considered, namely, the percentage of mottled fruit produced in the field by inoculated susceptible and resistant varieties during the course of the growing season. The percentages of mottled fruits in both varieties were negligible during the first 3 pickings.

TABLE 1.—*Average yield of susceptible (National Pickling) and resistant (Ohio MR17) varieties of cucumber in field experiments at Madison, Wisconsin*

Variety	Lb. of fruit per 25-ft. row in year indicated[a]				
				1954[b]	
	1951	1952	1953	Early planting	Late planting
National Pickling	26	74	73	36	23
Ohio MR17	92	106	68	47	55
LSD 19:1		8	NS	NS	15

[a] Plots were not replicated in 1951. In 1952, 1953, and 1954 they consisted of 25-ft. plots in 4 randomized replicate blocks.

[b] Early planting, May 19; late planting, June 25.

TABLE 2.—*Percentages of mottled fruits in National Pickling (NP) and Ohio MR17 (MR17) varieties in 4 successive weeks of August of 1952, 1953, and 1954 (field experiments)*

Week in August	Percentage of mottled fruits in year indicated					
	1952		1953		1954	
	NP	MR17	NP	MR17	NP	MR17
First	70	6	4	0	11	2
Second	96	16	16	6	30	7
Third	100	17	52	2	83	19
Fourth	99	30	64	1	80	41

The fruits picked during August, 1952, 1953, and 1954, were separated into mottled and unmottled classes (Table 2). Each year, the proportion of mottled fruits in the susceptible variety increased as the season advanced. The increase was most rapid in 1952 and least rapid in 1953. Whereas the percentage of mottled fruits in the resistant variety increased to 30 and 41 per cent in 1952 and 1954, respectively, the true difference between varieties was even greater than the data indicate. Practically all diseased fruits of the resistant variety were very slightly mottled, whereas the percentage of severely mottled fruits in the susceptible variety increased as the season progressed.

In 1955, the variety National Pickling was compared with Ohio MR17, Ohio MR25, and Wisconsin SMR12, the last 3 being commercial resistant varieties. The mottled fruits were divided into 2 classes, designated as mildly mottled and severely mottled (Table 3). In this season all fruits of NP were severely mottled, whereas only 6 per cent of MR17 fruits were in that class. With the other 2 varieties, no fruits were severely mottled and the percentage of mildly mottled fruits was slightly lower than for MR17. Those fruits in the mildly mottled class were acceptable for processing. The percentage of mottled fruit was considered a more reliable criterion than was yield for judging the relative resistance of varieties and of individual plants.

GREENHOUSE EXPERIMENTS.—When National Pickling (susceptible) cucumber seedlings in the second- or third-leaf stage were inoculated on the cotyledons in the greenhouse at temperatures favorable for growth of the host (20°–28°C), epinasty of the first leaf usually appeared in 4 or 5 days, and this was followed in 1–2 days by mottle and severe stunting. This is in

TABLE 3.—*Percentage of mildly and severely mottled fruits during the 1955 season in the National Pickling (susceptible) variety and in 3 resistant varieties (field experiment)*

Variety	Total fruits	Percentage mildly mottled	Percentage severely mottled
National Pickling	64	0	100
Ohio MR 17	150	35	6
Ohio MR 25	193	7	0
Wisconsin SMR 12	136	13	0

TABLE 4.—*Ratios of height of inoculated to that of noninoculated plants of susceptible (NP) and resistant (MR17) varieties 21 days after inoculation in the greenhouse*[a]

Series	Date of inoculation	Ratios at indicated temperature and with indicated variety							
		16°C		20°C		24°C		28°C	
		NP	MR17	NP	MR17	NP	MR17	NP	MR17
1	Dec. 2	0.73	0.91	0.63	0.62	0.55	0.62	[b]	0.66
1a	Dec. 5	0.68	0.65	0.69	0.82	0.52	0.75	0.58	0.79
2	Jan. 20	1.11	[c]	0.87	0.71	0.59	0.59	[b]	0.80
3	Feb. 10	1.02	0.88	0.60	0.77	0.55	0.83	0.49	0.68
4	April 2	0.92	0.76	0.53	0.76	0.59	0.85	0.52	0.77

[a] Ratios were determined from heights of 10 noninoculated and 10 inoculated plants in each treatment.
[b] Plants were killed by mosaic at 14 days.
[c] Plants killed by damping-off organisms.

contrast to the appearance of the disease in inoculated plants in field plots where initial symptoms were usually delayed until 4–6 weeks after inoculation. Although similar differences were recognized as early as 1920 by Doolittle[4], no adequate explanation for such differences has been found. When MR17 (resistant) cucumber seedlings were treated similarly in the greenhouse during midwinter, when days were short and light intensity was low, much the same rate of progress and degree of severity followed. The possibility of assaying for resistance under natural midwinter greenhouse conditions obviously was remote.

In order to determine what conditions, if any, could be provided in the greenhouse for a satisfactory assay of resistance, the 2 varieties (NP and MR17) were inoculated in the seedling stage and maintained in constant-temperature houses without supplementary light at 16°, 20°, 24°, and 28°C. In each series, 10 inoculated and 10 noninoculated plants of each variety were used. Four successive series were run, inoculations being made on December 2 (series 1), January 20 (series 2), February 10 (series 3), and April 2 (series 4). Each series was run for 21 days. In each successive series, day length and light intensity increased. In a subsequent season, series 1 was repeated except that normal light was supplemented by flourescent lights for a 15-hour period during each day (series 1a).

Symptoms always appeared first at 28°C (4–6 days after inoculation); they appeared 1 or 2 days later at 24° and 1–7 days still later at 20°. At 16°, symptoms in series 1 and 4 appeared 6–7 days later than at 20° and remained mild throughout the experiments; in series 2 and 3 no symptoms appeared throughout the 21-day period. There was practically no difference between varieties in the time of appearance of symptoms. Although initial mottle symptoms could not be used to distinguish clearly between resistant and susceptible varieties, resistant plants in series 3 and 4 tended to recover from the initial shock phase of the disease and to continue to grow with little further expression of severe mottle of young leaves as they developed. This tendency was distinct at 24° and 28° but barely noticeable at 16° and 20°.

At the end of each series, the average heights of noninoculated and inoculated plants were measured and the ratios of the latter to the former determined (Table 4). In both varieties in each series, growth of noninoculated plants increased with temperature from 16° to 28°C. The suppression of growth was usually, but not always, greater in NP than in MR17 in any given treatment; however, the differences in the ratios of the 2 varieties were small at all 4 temperatures in series 1 and 2. The differences were most marked and most consistent in series 3 and 4 at 20°, 24°, and 28°. It was apparent, therefore, that as day length and light intensity increased, as between series, the difference between varieties widened. It was also evident that after February 10 the difference widened as temperature increased. Additional experiments were conducted the following year at approximately the same time intervals. Confirmatory results were obtained in all cases. These results indicated that under Madison greenhouse conditions, the best environment for distinguishing resistant from susceptible plants was that in which day length and light intensity were the greatest and when temperature was most favorable for cucumber plant growth and for disease development. When light intensity was lowest and day length was shortest, optimum temperature was insufficient to bring out expression of resistance (series 1). When light intensity and day length were increased in midwinter (series 1a), however, essentially the same results were secured as in April (series 4) without supplementary light.

Since the experiments showed that the best expression of resistance in the greenhouse was obtained by determining the height ratios of inoculated to noninoculated plants grown under near-optimum conditions of temperature, light intensity, and day length, a final set of experiments was run during midsummer with 3 susceptible and 3 resistant varieties. One series was inoculated on June 3, another on July 13. The height ratios (Table 4) at 21 days showed distinct differences between the resistant and susceptible

[4] Doolittle, S. P. 1920. The mosaic disease of cucurbits. U. S. Dept. Agr. Bul. 879, 69 p.

TABLE 5.—*Ratios of height of inoculated to that of noninoculated plants of resistant and susceptible cucumber varieties when inoculations were made during summer months in the greenhouse*[a]

Variety	Ratios 21 days after indicated date of inoculation	
	June 9	July 13
Chicago Pickling (susceptible)	0.40	0.44
National Pickling (susceptible)	0.47	0.37
Early Cluster (susceptible)	0.43	0.45
Kanmore (resistant)	1.10	0.65
Ohio MR17 (resistant)	0.52	0.61
Yorkstate Pickling (resistant)	0.65	0.72

[a] Ratios were determined from heights of 10 inoculated and 10 noninoculated plants in each case, 21 days after inoculation.

groups. It is obvious, however, that the differences were much less under the best greenhouse conditions tested than in the mottled-fruit assay used earlier under field conditions.

DISCUSSION.—The mosaic-resistant pickling varieties of cucumber now in general use are not completely resistant to cucumber mosaic virus. They become infected readily, but under field conditions they yield satisfactorily and produce a negligible amount of severely mottled fruits at the pickling stage. The percentage and degree of mottling of fruits are the best criteria for judging resistance in the field. Since plants of susceptible and resistant varieties react quite differently in the greenhouse than in the field, other means of distinction between the 2 classes are necessary for greenhouse assay. In midwinter, with short day length and low light intensity, the disease effects on resistant and susceptible plants are very similar; with artificial lighting, however, some differences can be determined. During spring and summer months, with temperatures averaging 24°–28°, a fairly reliable distinction between resistant and susceptible plants may be made on the basis of relative suppression of growth and the tendency of resistant plants to recover from the early shock phase of the disease. The greenhouse assay, however, is considered to be useful only as a supplement to field evaluation. It has not been possible to evaluate the degree of resistance by determining the relative distance from the inoculated cotyledons to the true leaf on which symptoms first appear, as suggested by Shifriss et al.[5]

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[5] Shifriss, O., C. H. Myers, and C. Chupp. 1942. Resistance to mosaic virus in the cucumber. Phytopathology 32: 773-784.

PHYTOPATHOLOGICAL NOTES

In Vitro Development of Resistance to an Antibiotic by Two Plant Pathogenic Bacteria. J. CARMONA-GOMEZ.[1] The phenomenon of acquired resistance to antibiotics *in vitro* by bacteria pathogenic to humans has been reported many times in the literature. An extensive review of the literature on this subject is given by Chain et al.[2] Unfortunately, very little work has been done on the development of resistance to antibiotics by plant pathogenic bacteria. One explanation for this may be that investigations of the effects of antibiotics on plant pathogens have been made only in recent years. Zaumeyer et al[3] found that some mutants of the common blight organism, *Xanthomonas phaseoli* (E. F. Sm.) Dows., tolerated concentrations of streptomycin as high as 0.1 per cent. No such mutants of the halo blight organism, *Pseudomonas phaseolicola* (Burk.) Dows., were found. English and VanHalsema[4] developed strains of 2 plant pathogenic bacteria resistant *in vitro* to streptomycin, to terramycin, and to combinations of both antibiotics. They used *Erwinia amylovora* (Bur.) Winsl. et al, the causal agent of fire blight in pears and apples, and *Xanthomonas vesicatoria* (Doidge) Dows., the bacterium that causes the pustule spot on pepper.

The objective of the present work was to determine whether there could be developed strains of *Xanthomonas phaseoli* var. *fuscans* (Burk.) Starr & Burk., and *Xanthomonas vignicola* Burk., resistant to the antibiotic A-6 (a crude sample of a basic antibiotic produced by an unidentified streptomycete; distributed by Wyeth Laboratories Inc.). This material was selected because it was found to be fairly active against these bean pathogens when tested *in vitro* by the meth-

[1] Part of a thesis presented to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science.

[2] Chain, E., H. W. Florey, and M. A. Jennings. 1949. Acquired resistance of micro-organisms to penicillin. In Antibiotics. v. 2. Oxford University Press, London. p. 1111-1136.

[3] Zaumeyer, W. J., H. R. Thomas, and J. W. Mitchell. 1953. Streptomycin controls halo blight. Ann. Vegetable Grower 1 (11): 5, 16.

[4] English, A. R., and G. VanHalsema. 1954. A note on the delay in the emergence of resistant Xanthomonas and Erwinia strains by the use of streptomycin plus terramycin combinations. U. S. Dept. Agr. Pl. Dis. Rptr. 38: 429-431.

FIG. 1. Zones of inhibition by paper discs that had been immersed in sterile solutions containing A) 25 μg, B) 50 μg, C) 100 μg, and D) 200 μg of the antibiotic A-6 per ml and then placed on agar plate seeded with *X. phaseoli* var. *fuscans*.

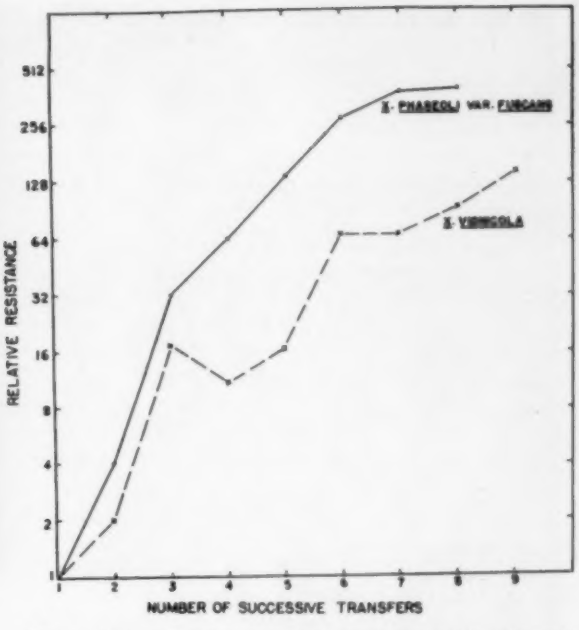

FIG. 2. Induced resistance of *X. vignicola* and *X. phaseoli* var. *fuscans* to A-6 after exposure to increasing concentrations of the antibiotic.

od described by Vincent and Vincent[5] (Fig. 1).

The initial concentration of the antibiotic in nutrient broth at which *X. phaseoli* var. *fuscans* would grow was approximately 0.75 μg/ml, that for *X. vignicola* was 3 μg/ml. A 2-fold dilution series of sterile Bacto broth was used in 6 × 5/8-in. test tubes. One-tenth ml of medium taken from the highest concentration at which growth of the bacteria was readily visible was used as inoculum for the next series. This was continued through several transfers, and in this way resistance of the organisms to the antibiotic was increased gradually. A sterile technique was used throughout. All cultures were kept at 30°C, and new transfers were made to higher concentration at the end of 8–10 days. The duration of the experiment was 10 weeks and covered observations made on 8 transfers for *X. phaseoli* var. *fuscans* and 9 transfers for *X. vignicola*.

With *X. phaseoli* var. *fuscans*, each successive transfer resulted in growth at progressively higher concentrations of A-6, up to a concentration of 300 μg/ml following the eighth transfer (Fig. 2). This is 400 times greater than that tolerated by the original culture.

The increase in resistance was not as regular with *X. vignicola* as it was with the other organism, but the ninth transfer resulted in growth in media containing 400 μg/ml. This is 133 times as much A-6 as was tolerated by the original culture of *X. vignicola*.

[5] Vincent, J. G., and H. W. Vincent. 1944. Filter paper disc modification of the Oxford cup penicillin determination. Soc. Exp. Biol. and Med. Proc. 55: 162-164.

If resistant strains of bacterial pathogens were to develop in the field to an extent similar to that reported here, serious problems would confront plant pathologists. Recommendations of concentrations and/or kind of toxicants for use against an organism would have to be amended continuously. The results of this experiment also might point to the reason why some antibiotics seemed to give satisfactory control in early experiments but later failed to give satisfactory control in field tests.—Department of Plant Pathology, Cornell University, Ithaca, N. Y.

Heat-induced Susceptibility of Beans to Some Viruses and Fungi. C. E. YARWOOD. When bean (*Phaseolus vulgaris* L.) leaves were immersed in hot water for a few seconds before inoculation, their susceptibility to tobacco mosaic virus (TMV), spotted wilt virus, peach yellow bud mosaic virus, apple mosaic virus, the bean rust fungus (*Uromyces phaseoli* (Pers.) Wint.), the bean mildew fungus (*Erysiphe polygoni* DC.), and the bean anthracnose fungus (*Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav.) was increased. Heat treatments after inoculation also increased infection in many cases. This extends previous information on heat-induced susceptibility to plant pathogens.[1,2,3]

[1] Kassanis, B. 1952. Some effects of high temperature on the susceptibility of plants to infection by viruses. Ann. Appl. Biol. 39: 358-369.

[2] Bawden, F. C. 1955. Virus diseases in plants. Jour. Roy. Soc. Arts. 103: 436-451.

[3] Yarwood, C. E. 1952. The phosphate effect in plant virus inoculations. Phytopathology 42: 137-143.

Plants with twin primary unifoliate leaves were used 9–19 days from planting of seed in 4-in. pots in the greenhouse at about 21°C. One leaf of each plant was unheated and served as a control. The opposite attached leaf was immersed in water at 40°, 45°, 50°, or 55°C before or after inoculation and then surface dried. Both leaves were similarly inoculated with the test pathogen.

After infection appeared, disease lesions were counted, and the number of lesions on the heated leaf was divided by the number of lesions on the control leaf to give an infection quotient. For example, in a representative trial, 21 and 11 TMV lesions resulted on duplicate control leaves, and 153 and 89 lesions, respectively, resulted on the opposite leaves that had been heated at 45°C for 60 seconds before inoculation. The infection quotients were then 5.9 and 8.1, respectively, and averaged 7.0.

Typical infection quotients for heat treatments before inoculation for different pathogens on different bean varieties were as follows: TMV on Pinto (as above)—7.0; spotted wilt virus on Pinto (60 seconds at 45°)—2.3; peach yellow bud mosaic virus on Pencil Pod (60 seconds at 45°)—6.2; *C. lindemuthianum* on Dwarf Horticultural (10 seconds at 50°)—11.6; *E. polygoni* on Pinto (8 seconds at 50°)—3.9; and *U. phaseoli* on Pinto (30 seconds at 45°)—1.5. With the fungus infections studied, the increased susceptibility due to heating the leaves before inoculation was greatest on leaves that had become somewhat resistant because of age.

Infection quotients were infinite in the few cases where infection occurred on the heated leaves but not on the controls. Where infection occurred in the controls, the highest infection quotient was with *C. lindemuthianum* on Dwarf Horticultural inoculated at 19 days after the seed was planted. In 1 pair of this trial, the control leaf showed 1 lesion at 5 days after inoculation whereas the opposite leaf, which had been treated for 15 seconds at 50° before inoculation, showed 185 lesions. At 6 days after inoculation, the control leaf showed 12 lesions whereas the heated leaf was collapsed from numerous coalesced lesions. Greater increases in infection due to heating the leaves before inoculation have resulted with bean anthracnose than with any other disease studied.

Infection quotients approaching zero resulted from some treatments where the leaves were injured. In 1 trial, for example, 163 TMV lesions resulted on the 2 control leaves, whereas only 3 lesions resulted on the opposite leaves that had been treated for 10 seconds at 55° before inoculation.

Optimum temperatures and periods of heating have not been clearly determined. Increases in TMV infection on Pinto bean resulted from heating leaves for 100–600 seconds at 40° before inoculation, with a maximum increase at about 360 seconds. At 45°, 50°, and 55°, maximum increases in TMV infection resulted from heating at about 60, 10, and 1 second, respectively. For temperatures between 40° and 55°, the temperature coefficient for heat-induced susceptibility to TMV was therefore about 40 but has not been adequately determined.

The increased susceptibility to TMV due to the heating of leaves before inoculation lasted at least 3 days. In a representative test, the infection quotient for duplicate plants was 3.9 for inoculations made immediately after heating, 3.0 for inoculations at 35 hours after heating, and 2.6 for inoculations at 72 hours after heating. In this case the heating was for 60 seconds at 45°, consequently the treatment effect lasted several thousand times as long as did the treatment period.

Cold- or hot-water treatments immediately after inoculation reduced subsequent numbers of lesions of several virus infections,[4,5] but hot-water treatments given several hours after inoculation increased numbers of lesions in many cases. In a representative trial, duplicate untreated Bountiful bean leaves yielded 2 and 4 apple mosaic virus lesions, whereas the opposite leaves treated for 5 seconds at 50° at 17 hours after inoculation yielded 42 and 48 lesions, respectively. Other infection quotients for sample treatments after inoculation were 11 for a strain of tobacco necrosis virus on Pinto (15 seconds at 50° at 15 hours after inoculation) and 3.7 for TMV on Pinto (10 seconds at 50° at 12 hours after inoculation).

These hot-water treatments that increased numbers of lesions commonly increased the size of the lesions also. This was observed with TMV on Pinto for heat treatments before or after inoculation, with apple mosaic virus on Bountiful for heat treatments after inoculation, with *C. lindemuthianum* on Dwarf Horticultural for heat treatments applied before inoculation, with tobacco necrosis virus on Pinto for heat treatments after inoculation, and with *E. polygoni* on Pinto for heat treatments applied before inoculation. In a representative test, TMV lesions on control leaves averaged 210 μ in diameter, whereas lesions on leaves that had been heated for 20 seconds at 50° at 9 hours after inoculation averaged 690 μ. The lesions on the heated leaves were thus more than 10 times the area of those on the control leaves.

When inoculated leaves were dried quickly[5] after TMV inoculation following heat treatment, the increase in infection due to heat treatment was less than when the leaves were allowed to dry slowly. When the inoculum was suspended in 1 per cent K_2HPO_4 solution,[3] infection was usually less on heat-treated than on control leaves; however, heat treatments after inoculation increased the size of the lesions on leaves inoculated with inoculum containing phosphate. When the test plants were incubated in a moist chamber after inoculation,[5] the increase in infection due to heat-

[4] Dale, J. L., and H. H. Thornberry. 1956. The influence of pH, buffer, and temperature of the wash medium on the amount of infection by tobacco mosaic virus on Scotia bean leaves. (Abs.) Phytopathology 46: 10.

[5] Yarwood, C. E. 1955. Deleterious effects of water in plant virus inoculations. Virology 1: 268-285.

ing the leaves before inoculation was greater than when the plants were incubated in the dry air of the greenhouse.

Heat treatments applied before or after inoculation with viruses on hosts other than bean have not as yet resulted in definite increases in numbers of lesions or in size of lesions. Heavy infection but no increase in local lesion numbers due to heat treatment has been observed for tobacco necrosis virus on sunflower; for tobacco necrosis virus on cowpea (*Vigna sinensis* (Torner) Savi); for spotted wilt virus on squash (*Cucurbita maxima* Dcne.); and for peach yellow bud mosaic virus on squash, sunflower, and sugar beet (*Beta vulgaris* L.).

Sunflower rust (*Puccinia helianthi* Schw. on *Helianthus annuus* L.) was the only infection tested on a host other than bean where heating before inoculation clearly increased infection. For all paired comparisons in which the test leaves were treated at between 10 and 20 seconds at 50°, the treated leaves had more pustules than the controls in 14 out of 17 pairs, and the gross number of pustules counted was 1902 for the heated and 907 for the control.—Department of Plant Pathology, University of California, Berkeley.

Stripe Rust Resistant Mutants Obtained from Irradiation of Gabo Wheat.[1] C. F. Konzak, N. E. Borlaug, Aristeo Acosta, and John Gibler.[2] Gabo, a spring wheat variety of Australian origin, is widely adapted on the Pacific Coastal Plain of Mexico. This variety is also adapted in Central Mexico at elevations up to 6000 ft, but cannot be safely cultivated there because of its extreme susceptibility to stripe rust (caused by *Puccinia glumarum* (Schm.) Eriks. & E. Henn.). Since the climate of some of the higher valleys of Central Mexico favors the development of very severe epiphytotics of stripe rust nearly every summer, the field conditions in that region are ideal for the recognition and testing of any plants suspected as mutants for resistance to this disease.

Experiments were undertaken to attempt to produce rust-resistant mutants by irradiation of seeds.[3] Dry seeds of Gabo were irradiated with doses of 15,000r of X rays or about 2.3×10^{12} Nth/cm^2 thermal neutrons at the Brookhaven National Laboratory in 1953. First generation plants were grown in plots near Ciudad Obregon, Sonora, Mexico, and second-generation progenies from these plants were grown at the experimental station at Chapingo during the summer of 1954. Notes were not taken on stripe rust reaction during 1954 because infection was light in that year, but numerous agronomically typical and atypical Gabo plants were selected for further testing. The X_3 and N_3 progenies from the selected plants were grown at Chapingo during the summer of 1955, and in this year stripe rust was especially severe. One N_3 line bred true for a high degree of stripe rust resistance; otherwise it was apparently typical of the Gabo variety. Twelve additional progenies from the X-ray and neutron treatments showed segregation for various degrees of resistance. These resistant types were classified as 1, 2, and 3 on a scale of 0 to 5, in which 0 represented highly resistant reactions and 5 completely susceptible reactions; the reaction of the parental variety was 4++. No variants resistant to stripe rust were found among a large number of nonirradiated Gabo lines tested under the same conditions. The fact that a true-breeding third-generation line was obtained suggests that the mutation is recessive in inheritance and similar to the natural types. Since the

Fig. 1. Leaves showing stripe rust reaction. Left: mutant resistant to stripe rust, rust reaction type 1. Right: Gabo parent, rust reaction type 4++.

[1] Preliminary report on a cooperative project between Brookhaven National Laboratory and Rockefeller Foundation in Mexico through the Office of Special Studies of the Mexican Ministry of Agriculture. A portion of the work was carried out at the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[2] Respectively, Associate Geneticist, Brookhaven National Laboratory, Upton, L. I., N. Y.; Plant Pathologist, Rockefeller Foundation Mexican Program; Agronomist, Office of Special Studies del Secretario de Agriculture y Ganaderia; and Plant Pathologist, Rockefeller Foundation, now with the Colombian Agricultural program in Bogota, Colombia.

[3] Konzak, C. F. 1954. Stem rust resistance in oats induced by nuclear radiation. Agron. Jour. 46: 538-540.

line originated from a single N_2 plant, as did several susceptible sister lines, it is believed that the resistance was induced by the treatment. More extensive experiments are now under way to test this hypothesis. The reaction of one of the mutants, compared with that of the parent variety, is shown in Figure 1. These and many other similar observations offer considerable hope for a more general use of artificially induced mutations in practical plant breeding.—Brookhaven National Laboratory, Upton, New York.

Spectographic Analysis of Water Agar, Potato-dextrose Agar, and V-8 Juice Agar. PATRICK M. MILLER. Formulae for many biological media specify use of agar that has been purified of contaminating metallic ions by some manner, as by washing with pyridine. Yet there is little readily available information on what these ions might be or in what concentration they are present in water agar. In addition, comparison of the metallic ion contents of V-8[1] juice agar and potato-dextrose agar (PDA) might aid in determining the reason why growth of several fungi on V-8 juice agar was superior to that on PDA.[2,3] Hence, analyses of Difco bacto-agar (water agar), Difco potato-dextrose agar (PDA), and V-8 juice agar[2] were made with an ARL 1.5-m spectograph. The following results are expressed as ppm of each metal that would be present 1) in 2 per cent water agar; 2) in 3.9 per cent PDA; and 3) in V-8 juice agar containing 2 per cent water agar, 3 g of calcium carbonate, and 200 ml of V-8 juice per liter. Amounts (in ppm) found in water agar, PDA, and V-8 juice agar, respectively, are as follows: potassium—0, 312, 400; calcium—120, 120, 800; magnesium—24, 39, 40; phosphorus—8, 19, 36; sodium—20, 39, 102; manganese—0.0, 0.1, 0.2; iron—2.0, 3.9, 2.8; aluminum— 3.8, 5.1, 2.6; copper—0.08, 0.31, 0.40; and boron—2.6, 3.5, 2.8. Copper is present in PDA and V-8 juice agar in amounts adequate to be toxic to some fungi, particularly some of the Phycomycetes. The absence of potassium and manganese in water agar might explain the failure of some fungi to develop on this media. Calcium and sodium are more plentiful in V-8 juice agar than in PDA, but there are adequate amounts of calcium in PDA and the significance of sodium in the nutrition of fungi is doubtful. Hence, the superior nutritional properties of V-8 juice agar must reside in other than its metallic ion content.—Department of Plant Pathology and Botany, The Connecticut Agricultural Experiment Station, New Haven 4, Connecticut.

[1] Proprietary name of a product of the Campbell Soup Co.; a mixture of the juices of tomato, carrot, celery, parsley, beet, lettuce, spinach, and watercress.

[2] Diener, U. L. 1952. A method for inducing abundant sporulation of Stemphylium solani in pure culture. (Abs.) Phytopathology 42: 7.

[3] Miller, P. M. 1955. V-8 juice agar as a general-purpose medium for fungi and bacteria. Phytopathology 45: 461-462.

Storing Inoculum of Pseudomonas glycinea in Host Tissue by Freezing.[1] F. I. FROSHEISER.[2] Cultures of *Pseudomonas glycinea* Coerper isolated from infected soybean leaves and maintained on agar media were sometimes unsuitable for use in producing bacterial blight when inoculated onto soybean plants. Apparently the bacteria lose their virulence when kept on agar media. A method of maintaining virulent inoculum of the alfalfa wilt bacterium, *Corynebacterium insidiosum* (McCall.) H. L. Jens., by keeping naturally infected host tissue in a freezer has been described.[3] It seemed possible that the same procedure might be effective with *Pseudomonas glycinea.*

Soybean leaves naturally infected with *P. glycinea* were collected in the field in August, put in plastic bags, and placed immediately in a freezer maintained at approximately −18°C. In June of the following year, leaves were removed from the freezer, placed in tap water for 30 minutes, then comminuted in a Waring Blendor for 3 minutes. The suspended material was filtered through several layers of cheesecloth to remove particles that might plug a sprayer nozzle, diluted with more water, and sprayed onto 6-week-old soybean plants in the field. A heavy infection of blight resulted.

Inoculum was similarly prepared from infected leaves that had been kept in the freezer for 20 and 31 months. For the preparation of the inoculum, 10 leaflets of average size, on which bacterial lesions covered approximately 2 per cent of the leaf area, were used for each liter of water. Leaves of soybean plants in the 1-trifoliate-leaf stage were inoculated in the greenhouse. Abundant infection was obtained with the inoculum of both ages.

This method of storing inoculum of *P. glycinea* requires very little time and effort, and the bacteria retain their virulence for more than 2 years. It also is very possible that such inoculum would comprise more virulent biotypes than would pure cultures maintained on agar media.—Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota.

[1] Paper No. 3529, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Formerly Research Assistant, Department of Plant Pathology and Botany, University of Minnesota.

[3] Kernkamp, M. F., and G. Hemerick. 1952. A "deep-freeze" method of maintaining virulent inoculum of the alfalfa wilt bacterium, Corynebacterium insidiosum. (Abs.) Phytopathology 42: 13.